DOCTORAL THESIS

Understanding the role of Inflammation in Coronary Heart Disease Patients with and without Depression

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UNDERSTANDING THE ROLE OF INFLAMMATION IN CORONARY HEART DISEASE PATIENTS WITH AND WITHOUT DEPRESSION

by

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ABSTRACT

Coronary heart disease (CHD) and depression are very common and often co-existing disorders. The prevalence of depression among patients with CHD is considerably higher as compared to the general population. Depression exacerbates adverse cardiac outcomes in CHD patients increasing the risk of cardiovascular morbidity and mortality, besides worsening the psychological and social morbidity. Inflammation has been recognised to be involved in the association between these two debilitating disorders.

Therefore, the present PhD thesis aimed to evaluate inflammatory responses and to investigate the pathophysiological mechanisms underlying the inflammatory activation in CHD patients with and without depression by assessing the function of two important biological factors regulating inflammation: the hypothalamus-pituitary-adrenal (HPA) axis and the glucocorticoid receptor (GR). Serum C-reactive protein (CRP), and plasma and salivary cortisol were measured using commercially available ELISA kits. Gene expression of GR and inflammatory biomarkers were analysed by means of quantitative real time PCR. GR function was assessed *in vitro* in isolated peripheral blood mononuclear cells using the dexamethasone inhibition of lipopolysaccharide-stimulated IL-6 production method. Serum levels of kynurenine pathway of tryptophan metabolism metabolites were measured using high performance liquid chromatography.

CHD patients with depression showed higher CRP levels and IL-6 gene expression compared with CHD non-depressed. Both plasma cortisol levels and salivary cortisol awakening response were significantly lower in patients with depression when compared with CHD alone. The CHD depressed group exhibited a reduction in GR expression and function. Tryptophan levels were significantly lower in patients with depression who also showed an increased kynurenine/tryptophan ratio, which in turn was associated with an increased in 3-hydroxykynurenine level.

In CHD patients, depression was accompanied by elevated levels of inflammation in the context of HPA axis hypoactivity, GR resistance, and increased activation of the kynurenine pathway. Reduced cortisol bioavailability and attenuated glucocorticoid responsiveness due to decreased numbers and sensitivity of GR may lead to insufficient glucocorticoid signalling and thus elevation of inflammation in CHD patients with depression. An increased inflammatory response may in turn lead to a diversion of the kynurenine pathway towards the neurotoxic branch.

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ACE	Angiotensin-converting-enzyme inhibitor
ACTB	Beta-actin
ACTH	Adrenocorticotropic hormone
AD	Antidepressant
ASA	Acetylsalicylic acid
AUC	Area under the curve
AVP	Arginine vasopressin
BBB	Blood-brain-barrier
BDI	Beck depression inventory
BSA	Bovine serum albumin
B2M	Beta-2-microglobulin
CAA	Competing amino acid
CAR	Cortisol awakening response
CHD	Coronary heart disease
CIS-R	Clinical Interview Schedule-Revised
CNS	Central nervous system
CRH	Corticotrophin-releasing hormone
CRP	C-reactive protein
DBD	DNA binding domain
DEX	Dexamethasone
DMSO	Dimethylsulfoxide, Me2SO
DSM	Diagnostic and Statistical Manual
DST	Dexamethasone suppression test
E.coli	Escherichia coli
ELISA	Enzyme-linked immunosorbant assay
EPA	Eicosapentaenoic acid
ETOH	Ethanol
FCS	Fetal calf serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GC	Glucocorticoid
GR	Glucocorticoid receptor
GRE	Glucocorticoid responsive element
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
HRP	Horseradish Peroxidase
HSP	Heat Shock Protein
ICPE	International Consortium of Psychiatric Epidemiology
IDO	Indoleamine 2,3 dioxygenase
IL	Interleukin
KAT	Kynurenine aminotransferase
KMO	Kynurenine 3-monooxydase
KYN	Kynurenine
KYNA	Kynurenic acid
KYNU	Kynureninase
LPS	Lipopolysaccharide
MAOI	Monoamine oxidase inhibitor
MAPK	Mitogen activated protein kinase
MC2-R	Type 2 Melanocortin-receptor
MDD	Major depressive disorder
MI	Myocardial infarction
MR	Mineralocorticoid receptor
NAS	N-acetyl serotonin
NF-kB	Nuclear Factor-kappaBeta
NMDA	N-methyl-D-aspartate
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMP	Paramagnetic particles
PTSD	Post traumatic stress disorder

PUFA	Polyunsaturated Fatty Acid
PVN	Paraventricular nucleus
QOF	Quality and Outcomes Framework
QUIN	Quinolinic acid
RLU	Relative light unit
SGK1	Serum glucocorticoid regulated kinase 1
SNIR	Serotonin-norepinephrine reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressants
TDO	Tryptophan-2,3-dioxygenase
TMB	Tetramethylbenzidine
TNF-α	Tumour Necrosis Factor-α
TPH	Tryptophan-5- hydroxylase
TRD	Treatment resistance depression
TRP	Tryptophan
3-HAA	3-hydroxyanthranilic acid
3-HK	3-hydroxykynurenin
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
5-HTP	5-hydrohytryptophan

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Chapter 1 INTRODUCTION

1.1 Major Depressive Disorder (MDD)

1.1.1 Definition and Epidemiology of Depression

Major depressive disorder (MDD) or clinical depression is a very common mental disorder characterized by distinct low mood and persistent emotions of sadness, hopelessness, emptiness, or irritability; and accompanied by loss of interest to enjoyable activities and diminished ability to response to pleasurable stimuli (anhedonia). Patients with major depression display clinically significant behavioural, cognitive, and psychophysiological changes. The symptoms include low energy, appetite impairments (weight gain or loss), sleep disturbances (such as insomnia or hypersomnia), and alteration in sexual desire. In addition, depressed people experience poor memory and concentration, slowing of actions and speech, difficulties making decisions and engaging or holding conversations, and attention deficits. Crying, feelings of regret, worthlessness or inappropriate guilt, and recurrent suicide thoughts are other symptoms present in depression. According to American Psychiatric Association (APA) stated in fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) at least five of the symptoms of major depressive episodes must persist for a minimum of two weeks and nearly every day, to meet the diagnostic criteria for MDD considering that one of the symptoms must be either depressed mood or loss of interest/pleasure (APA, 2013, Belmaker and Agam, 2008).

Depression, in general, is a complex and heterogeneous condition with no one established aetiology, variable course, and inconsistent response to treatment (Belmaker and Agam, 2008, Stetler and Miller, 2011). Depression can be a chronic and highly episodic disorder. Some patients experience MDD only in a single episode with almost two-thirds being

recovered within first year. However, the majority of patients, almost more than 75%, develop recurrent episodes usually within two years of recovery (Mueller et al., 1999, Solomon et al., 2000). Based on the clinical presentation and neurovegetative symptoms, MDD has been defined into various subtypes. For instance, melancholic depression is markedly characterised by pervasive anhedonia, diurnal variation of mood being worse in the morning, psychomotor retardation, early morning waking, decreased appetite and weight loss (Uher et al., 2011). In contrast, depression with atypical features is mainly marked by mood reactivity, extreme lethargy and fatigue, interpersonal rejection sensitivity, hypersomnia, weight gain and increased appetite (Gold and Chrousos, 2002, O'Keane et al., 2012).

Amongst all psychiatric conditions, depression is the most prevalent one affecting 4% to 7% of the general population (Eaton et al., 2007, Kessler, 2002). While the prevalence of depression is about 6% in men at any one time and 7%-12% lifetime incidence risk, the rates are higher in women to up to 18% with the lifetime risk of 20%-25% (Kemp et al., 2003, Kessler, 2003). Results from the International Consortium of Psychiatric Epidemiology (ICPE) Surveys revealed that being female and unmarried are the two major consistent socio-demographics that correlate with depression (Andrade et al., 2003). Depression is a universal human condition which affects individuals of all ethnicities and cultures. The disorder is also observed in all age groups. In the past, depression was mainly considered as a disorder of adulthood. However, after large evidence from epidemiological studies including adolescents as well as improvement in diagnoses of depression in children, it is now known that in many cases depressed adults experienced their first depressive symptoms in their adolescence (Burke et al., 1990, Kessler, 2002). With a growing elderly population, late-life depression is also a cause of concern. Specifically because the elderly tend to escape the strict criteria of the DSM of

clinical depression, since they often only display some symptoms of depression that clinically are believed to be associated with increased risk of disability and mortality in this population (Beekman et al., 2002, Kohn and Epstein-Lubow, 2006, Mulsant and Ganguli, 1999).

1.1.2 Depression in the Medically-ill

While depression affects millions of people worldwide, the prevalence rate greatly increases when comorbid with chronic physical conditions and medical illnesses. Prolonged physical illnesses such as asthma, diabetes, arthritis, heart disease, cancer, Alzheimer's and Parkinson's diseases are all associated with a high risk of comorbid depression (Cowles et al., 2009, Moussavi et al., 2007). Studies argue possible mechanisms underlying comorbidity of chronic medical conditions and depression such as physiological mechanisms, behavioural and environmental factors, and/or common genetic vulnerabilities (Licinio et al., 2002, Voinov et al., 2013). Many lines of evidence also suggest there is an association between medications and therapies related to medical illnesses and increased risk of developing depression. For example, interferon-α (INF-α) treatment for hepatitis C and cancer has been shown to be associated with development of depressive symptoms (Dieperink et al., 2003, Hauser et al., 2002, Wichers et al., 2005).

Depression has a huge impact on medically ill patients' health status leading to poorer quality of life and loss of productivity, and also increasing the risk of illness complications and death (Carney and Freedland, 2003, Noel et al., 2004). In addition, medically ill patients with comorbid depression are less able to cope with their physical symptoms related to their condition than patients without depression. In fact, depression seems to adversely affect the adaptation process in these patients (Cowles et al., 2009).

Furthermore, poor adherence to medication is another challenging issue associated with comorbidity of depression in patients with chronic illnesses (Grenard et al., 2011). Moreover, severe consequences of comorbid depression not only affect the health and social welfare of the patient as an individual, but also their families as well as having substantial economic and social impact on the wider community (Louch, 2009, Moussavi et al., 2007).

Unfortunately, depression in medically ill patients is generally under-recognized and consequently under-treated by health organizations. In fact, the presence of depressive symptoms is sometimes considered to be an understandable psychological reaction and natural response to the long term medical disorder the patient is dealing with. Thus, depression often can be normalized by the general practitioners, one of the main barriers in the management of depression in patients with chronic conditions (Coventry et al., 2011, Cowles et al., 2009).

1.1.3 Heart Disease and Depression

Heart disease and depression are two very common and often co-existing disorders affecting the population worldwide. According to the World Health Organization Global Burden of Disease Survey, coronary heart disease (CHD) and major depression are currently the first and second causes of disability in developed countries, and it is estimated that this will apply to all countries throughout the world by the year 2020 (Blazer, 2000, Licinio et al., 2002). The prevalence of depression among patients with established CHD is considerably higher as compared to the general population (Carney and Freedland, 2003). More than 50% of all heart disease patients experience symptoms of depression. Approximately, 17%-27% of outpatients and 35%-70% of inpatients with

CHD meet criteria for major depression compared to 4%-7% in the general population (Carney and Freedland, 2008, Rudisch and Nemeroff, 2003, Rutledge et al., 2006). In a study by Polsky et al., following up patients for eight years after new diagnosis of a medical illnesses, heart disease patients were found to have consistently higher risk for development of depressive symptoms compared to patients diagnosed with chronic lung disease, arthritis, diabetes, and cancer (Polsky et al., 2005).

In addition to psychological and social morbidity, depression exacerbates adverse cardiac outcomes in CHD patients. The study by Frasure-Smith and colleagues in 1993 introduced major depression as a significant predictor and risk factor for mortality in patients hospitalised following myocardial infarction (MI) (Frasure-Smith et al., 1993). Indeed, after several epidemiological and meta-analysis studies since then, depression is now recognized as a negative prognostic indicator and an independent factor greatly increasing the risk of cardiovascular related morbidity and mortality, regardless of aetiology and other known cardiac risk factors (Jiang et al., 2002, Meijer et al., 2011, Miller et al., 2002). However, the pathophysiological mechanisms underlying the increased incidence of depression in patients with CHD are yet to be understood, despite the severe consequences for the patients' health.

The relationship between heart disease and depression is bidirectional and multifaceted (Lippi et al., 2009). The mechanisms associated with the link between the two diseases are not completely understood and identifying the underlying mechanisms remains a big challenge (Whooley and Wong, 2013). Various genetic and biological approaches suggested the possibility of shared genetic substrates (see page 191) and/or outcome of common biological pathways (see page 53, 195) underlying the relation (Licinio et al., 2002).

Despite the findings in regards to bidirectional association between these two devastating disorders, and that the depression is a robust and independent risk factor for heart disease, both aetiologically and prognostically, clinical recognition of depression in heart disease patients is generally complex and a significant concern. Indeed, depression in heart disease patients in more likely to be under-diagnosed compared with those who only suffer from depression (Jiang et al., 2002). This brings the importance of screening and monitoring of depression in patients with heart disease, applying appropriate treatment plan, and whether the treatment of depression would ease and improve the prognosis of their cardiac condition.

Up-to-date there is no one established mechanism that can explain the increased incidence of depression in heart disease. There seems to be, however, shared vulnerability factors between these two disorders. Identification of biomarkers that predict future development of depressive symptoms in heart disease patients would help in the early diagnosis and treatment of depression, and could potentially decrease the early morbidity and mortality associated with depression in this population. This would be in turn possible through identifying exact pathways underlying the association between these two common mental and physical disorders.

Therefore, the present PhD thesis focuses on the potential mechanisms underlying the pathophysiology of depression in heart disease patients trying to advance further the current understanding of the link between these two debilitating conditions by investigating the possible biological pathways involved and exploring the consequences of depression occurrence in this specific population.

In the next section, major theories proposed the behind pathogenesis of depression will be described followed by a review of current knowledge in relation to the mechanisms associated with development of MDD in general that would in turn help us to better understand the disorder in the context of CHD, as well as the link between the two conditions.

1.2 The Biological Basis of Depression

1.2.1 Major Theories Behind Pathogenesis of Depression

There have been mounting lines of investigations trying to understand the biological mechanisms underlying the causality and pathogenesis of major depression. In spite of various theories proposed, there is no one established mechanism associated with depression due to the complexity and heterogeneous nature of this disorder. Some of the main approaches in this regard are being mentioned in the following paragraphs.

The genetic approach elucidates the heritability of depression considering the evidence of specific associated genes, dependent or independent heritable depression prone personality traits, and also family history of depression as one of the strong predictor (Kendler et al., 2006, Levinson, 2006). However, heritability of MDD is only moderate and to develop depression environmental factors and major life stressors interact with genetic vulnerability (Firk and Markus, 2007).

The monoamine-deficiency hypothesis as one of the major theories of depression claims depletion in monoamine neurotransmitters serotonin and norepinephrine in the brain in depressed people (Schildkraut, 1965). The hypothesis attempted to explain the efficacy of early antidepressants: tricyclic antidepressants and monoamine oxidase inhibitors, which increase the availability of the monoamine neurotransmitters in the synapse (Belmaker and Agam, 2008, Kalia, 2005). More recent studies have shown that it is in fact the interaction between serotonin reduction with other neurotransmitters and their associated proteins that is implicated in the pathophysiology of depression rather than just the serotonin deficiency (Kalia, 2005). Furthermore, alterations in serotonin receptor

expression and function are also implicated in MDD (López-Figueroa et al., 2004, Pierz and Thase, 2014).

Another approach in understanding the biological basis of depression is coming from studies on communication between the endocrine and immune systems and the brain, and indeed the association between immune alteration and depression (Blalock, 1994, Hopkins and Rothwell, 1995). The macrophage theory of depression proposed by Smith hypothesized excessive secretion of macrophage monokines as a cause of depression (Smith, 1991). In addition, the hypothalamic-pituitary-cortisol hypothesis suggests the alteration in cortisol response to stress as an underlying mechanism of pathophysiology of depression (Belmaker and Agam, 2008).

In fact, the more recent psychiatric approaches on mechanisms involved in the pathogenesis of MDD are focusing on the developments in psychoneuroimmunology research considering the neural-immune interaction and involving the role of inflammatory processes. The theory is now known as cytokine hypothesis of depression developed by consistent findings of elevated levels of pro-inflammatory cytokines, and their action as neuromodulators, in MDD patients (Schiepers et al., 2005). The latest theory has led to the inflammatory and neurodegenerative hypothesis by Maes et al. stating that inflammatory processes are associated with depression leading to the diminished neurogenesis and increased neurodegeneration observed in MDD patients (Maes et al., 2009).

Taking into the consideration all the theories mentioned above, this PhD thesis, has particularly focused on the role of inflammation in the pathogenesis of depression trying to better understand the pathophysiological mechanisms underlying inflammatory

activation, their association with adrenocortical and serotonergic system, and their consequences as a comorbid condition. The following sections describe the main physiological systems responsible for the regulation of immune and inflammatory response and their involvement in pathophysiology of depression.

1.2.2 Hypothalamic-Pituitary-Adrenal (HPA) Axis

Involvement of the hypothalamic-pituitary-adrenal (HPA) axis in depression has been reported consistently by various studies (Pariante and Miller, 2001). HPA axis is a main physiological circuit which connects the brain with the endocrine system and plays a fundamental role as a regulatory system in stress responses. In response to psychological, physical, and/or environmental stress stimuli, HPA system (Figure 1.1) is activated by stimulation of hypothalamus secreting corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) peptides secreted by the parvocellular neurons of the hypothalamic paraventricular nucleus (PVN). These neuropeptides exert their effect on the pituitary gland promoting the release of adrenocorticotropic hormone (ACTH) from the anterior lobe of the gland into the bloodstream. Circulating ACTH in turn triggers the biosynthesis and secretion of glucocorticoids (GCs) from the adrenal cortex via acting on type 2-melanocortin receptor (Breedlove et al., 2007, McQuade and Young, 2000).

GCs, cortisol (mainly in human and primates) and corticosterone (mainly in rodents), are the final product of the HPA axis. These steroid hormones are synthesized from cholesterol and exhibit a critical role in restoring and maintaining bodily stress-related homeostasis, modulating neuroendocrine and immune responses, regulating energy metabolism and inflammatory reactions, and influencing cardiovascular function (Breedlove et al., 2007, McQuade and Young, 2000).

The physiological function of the endogenous GCs, both central and peripheral effect, is mediated upon their interaction with specific intracellular receptors expressed in target tissues including the HPA axis itself where they play a critical role in maintaining the intrinsic homeostasis of the axis activity. In fact, the receptors facilitate regulation of HPA axis activity through GC-mediated negative feedback mechanisms inhibiting the secretion of ACTH from the pituitary as well as CRH from the hypothalamus (De Kloet et al., 1998, de Kloet et al., 2005).

Due to the central role of the HPA axis in the regulation of stress responses, and extensive evidence indicating the involvement of the system in the pathophysiology of stress-related disorders including MDD that will be discussed shortly in section 1.2.4 , in this PhD thesis, HPA axis is assessed by measuring the concentrations of circulating cortisol in plasma and also free cortisol levels in saliva. Disturbances in the HPA axis activity could be reflected in alterations in the levels of cortisol as the end product of the circuit that is investigated in this PhD thesis together with assessing the function of their specific receptors which is described in the next section.

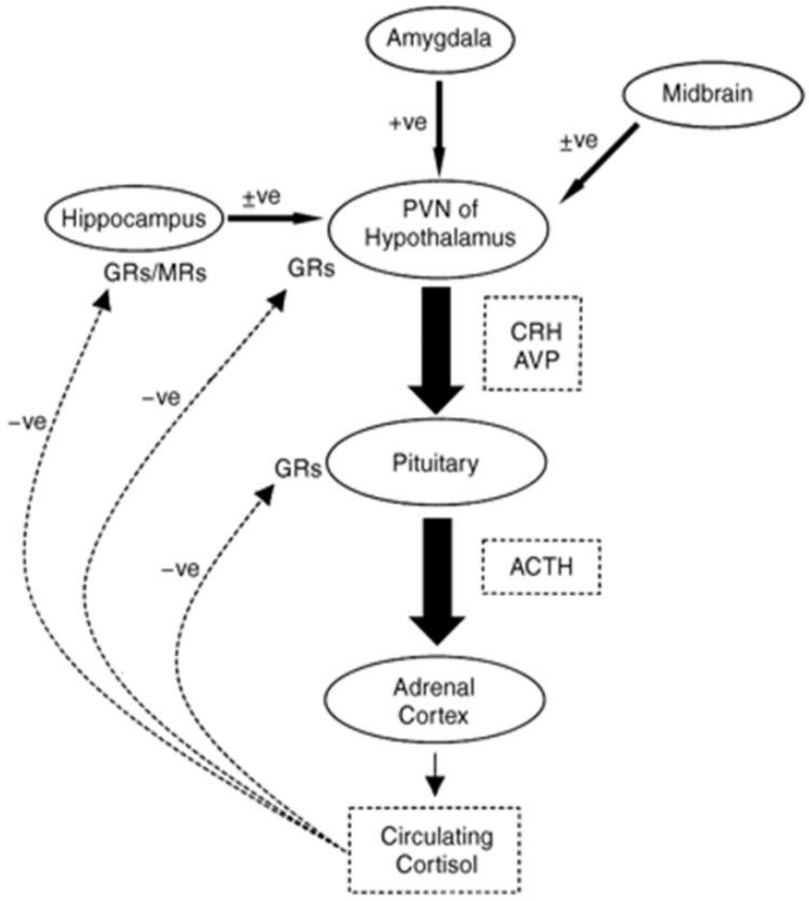

Figure 1.1 Schematic Diagram of the HPA Axis and Glucocorticoid Mediated Negative Feedback Regulation (McQuade and Young, 2000).

In response to the stress stimuli, hypothalamic-pituitary-adrenal (HPA) axis is activated by stimulation of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) secreted by hypothalamic paraventricular nucleus (PVN) which receives neuronal inputs from various brain regions including hippocampus, amygdala, and midbrain. CRH and AVP then promote the release of adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH in turn triggers the secretion of glucocorticoids (GCs) from the adrenal cortex. GCs, cortisol in human, are the final product of the HPA axis, and mediate their effect upon their interaction with glucocorticoid receptors (GRs). The specialised receptors are expressed in target tissues including the hippocampus and the HPA axis tissues where they facilitate regulation of HPA axis activity through GC-mediated negative feedback mechanisms by inhibiting the secretion of ACTH from the pituitary as well as CRH from the hypothalamus.

1.2.3 Glucocorticoid Receptor (GR)

There are two distinct steroid hormone receptor subtypes: type I or mineralocorticoid receptor (MR) and type II or GC receptor (GR) that are involved in the feedback regulation of the HPA axis. These specialised intracellular ligand-binding receptors are the members of the superfamily of nuclear transcription factors, and are involved in modulating the transcription of target genes. Under normal physiological conditions, MR is expressed in heart, intestine, and renal tissue as well as limbic brain regions, while GR is found in almost all tissues and organs in the human body (Anacker et al., 2011a, Marques et al., 2009).

Compared to MR, GR has lower affinity for endogenous corticosteroids such as cortisol, and it is activated when the GC levels are high. GR also shows high affinity for dexamethasone which is a synthetic cortisol, and only binds to GR and not to MR. While MR is mainly associated with circadian regulation of cortisol, GR plays a role in the modulation of peak morning response. In addition, GR activity appears to be more crucial in regulating stress-related responses when specifically there are elevated levels of endogenous GCs (De Kloet et al., 1998, Joels et al., 2008, Juruena et al., 2004, Pariante, 2004).

As illustrated in Figure 1.2, according to the Nucleocytoplasmic Traffic Model of GR (Juruena et al., 2004), inactivated GR primarily resides in the cytoplasm. The unbound cytosolic receptor is stabilized within an assembly of chaperone proteins such as heat shock proteins (HSPs): HSP56 and HSP90, and immunophilins. (Grad and Picard, 2007). GR is a ligand dependent transcription factor and is activated following binding to GC which passively diffuses across the cell membrane. Upon activation, GR undergoes conformational changes, dissociates from HSPs, and translocate to the nucleus where it

regulates gene expression. In fact, GR positively or negatively alters gene transcription either directly by interaction of its DNA binding domain with particular promoter sequence of the DNA known as GC responsive elements (GREs), or indirectly via binding with other transcription factors (protein-protein interaction) which in turn bind to the DNA response elements (Marques et al., 2009, Pariante and Miller, 2001).

GR sensitivity to GC is crucial in order to produce an appropriate response that is determined by the number, affinity, and function of the receptor including the ability of GR to bind the ligand, to translocate from cytoplasm into the nucleolus, and to interact with other signal transduction pathways (Marques et al., 2009).

Activation of gene expression by GR is known as transactivation in which GR stimulates transcription rate of a respective target gene, while negative alteration of gene expression by GR is called transrepression in which GR suppresses the other transcription factors activity. The crucial immunosuppressive and anti-inflammatory roles of GC are in fact mediated through GR-dependent transrepression which targets the genes associated with inflammatory cytokines including interleukins (Anacker et al., 2011a, De Bosscher and Haegeman, 2009). Pharmacologically, the gene-repressing properties of GR are targeted for treatment of inflammatory and autoimmune disorders using exogenous GCs (De Bosscher and Haegeman, 2009). However, the desired therapeutic use of these steroid agents is usually followed by adverse side effects mainly associated with gene-activating properties of GR (Schacke et al., 2002).

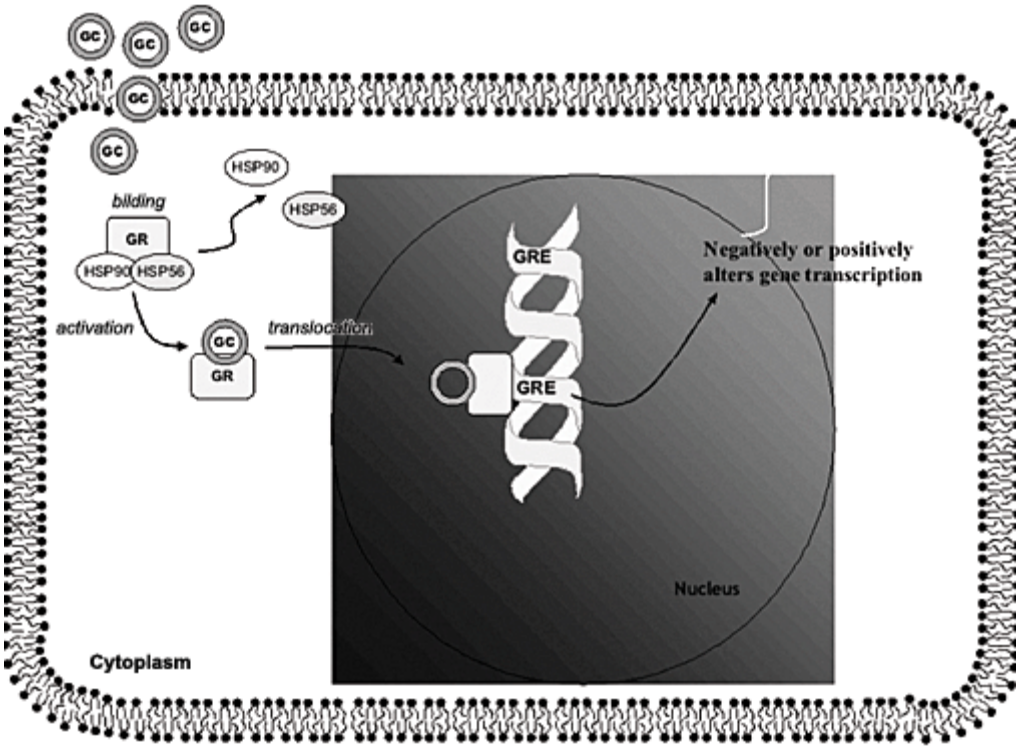

Figure 1.2 Schematic Representation of GR Activation and Function (Nucleocytoplasmic Traffic Model) (Juruena et al., 2004).

GR is primarily resides in the cytoplasm in an inactivated form and in a complex assembly of chaperone proteins such as heat shock proteins. GR is activated following binding to GC which passively diffuses across the cell membrane. Upon activation, GR undergoes conformational changes, dissociates from HSPs, and translocate to the nucleus where it negatively or positively alters gene transcription.

There are two homologous isoforms associated with human GR that are encoded by GR gene: GRα and GRβ. GRα is the cytoplasmic ligand-binding isoform, which is the classic GR and regulates gene transcription. In contrast, GRβ, which is the nuclear localized isoform, does not seem to bind to any ligand, but is known to affect GRα by attenuating its transcriptional activity. Elevation in inflammatory responses and specifically pro-inflammatory cytokines increase expression of the GRβ leading to accumulation of this relatively inert isoform that results in decreased in GRα function (Webster et al., 2001, Wichers and Maes, 2002).

Taking into consideration the crucial role of GR in negative feedback regulation of HPA axis and also its anti-inflammatory effect through transrepression activity, both expression and function of GR are assessed in this PhD thesis.

1.2.4 HPA axis, GR, and Depression

Generally, depressed patients tend to exhibit dysfunction of the immune system. The immune system disturbance in MDD patients is characterized by an overactivation of inflammatory responses (Empana et al., 2005, Raison et al., 2006) as well as a failure of the regulatory processes that maintain the balance, thus leading to a dysregulation in the control of inflammation (Holsboer, 2000, Pace et al., 2007, Pariante, 2006, Pariante and Miller, 2001). Disturbances in the HPA axis as a central regulatory system are implicated in the manifestations of psychosomatic and psychiatric disorders. HPA axis disfunctionality includes both hyperactivity and hypoactivity of the system leading to dysregulation of stress related responses (Kudielka et al., 2006, Raison and Miller, 2003).

Alteration of the HPA axis and elevated level of GCs are two well-documented features observed in a significant proportion of patients with major depression and are assumed to be involved in both aetiology and pathogenesis of the disease (Pariante and Miller, 2001, Plotsky et al., 1998). Indeed, almost half of the depressed patients show increased level of cortisol in cerebrospinal fluid, plasma and urine, consistent with overactivity of the HPA axis; and enlargement of pituitary and adrenal glands (Otte et al., 2004, Pariante and Miller, 2001, Stewart et al., 2009). However, the association between depression and the increased cortisol levels is mostly observed in older and/or more severely depressed individuals (Burke et al., 2005) and also in medically ill patients (Otte et al., 2004).

The hyperactivity of the HPA axis seems to reflect an impaired ability of GC hormones to exert their physiological effects (glucocorticoid resistance), including the negative feedback on the HPA axis itself as well as the anti-inflammatory effects at the peripheral level (Pariante, 2006, Pariante and Miller, 2001). In fact, the inflammatory response, which is regulated by the effect of GC hormones in normal conditions, cannot be terminated due to GR resistance and diminished sensitivity of immune cells to the hormones (Marques et al., 2009, Stark et al., 2001).

Evidence suggests that GC resistance is observed in more than half of the patients with major depression and is associated with dysfunction of negative feedback pathway which is normally regulated by the effect of cortisol suppressing CRH production. Therefore, one of the reasons for CRH hyperactivity is due to the resistance to the GCs inhibitory effects (Holsboer, 2000, Nemeroff, 1996). Indeed, it has been shown that MDD patients have HPA axis hyperactivity in the presence of GR resistance (Carvalho et al., 2008). Although the aetiology of presence of GC resistance found in depression is not known, some studies suggest that prolonged inflammation has a direct effect in reducing GR

sensitivity through the interaction of cytokine signalling pathway with GR signalling pathway, thereby disruption of functional properties of the receptor (Miller et al., 1999).

Alteration in GR function could explain impairment of the GC-mediated negative feedback regulation on HPA axis. In addition, a reduction in the number of GR has been also reported in the depressed patients (Pace et al., 2007, Pariante and Miller, 2001). Studies on therapeutic strategies using antidepressants report that some of these medications exert their effect directly on GR through increasing expression and modulating function of these receptors, hence the regulation of GR-mediated HPA axis activity. Since excessive inflammatory processes are involved in the pathogenesis of depression, some antidepressants also appear to exhibit beneficial effects through reducing levels of inflammatory biomarkers and modulating inflammation-induced depression (Carvalho et al., 2010, Pariante and Miller, 2001).

Despite the extensive evidence in regards to HPA axis hyperactivity and overproduction of GCs that can disrupt cellular functioning as well as damage to the brain structure and therefore association with stress related conditions, insufficient GC signalling is also believed to contribute to the pathogenesis of such disorders. The evidence suggests that inadequate signalling capacity of GCs could be not only due to GR-mediated signal transduction, but also reduced GC bioavailability itself (Raison and Miller, 2003).

Hypocortisolism has been observed in stress related disorders since 1960s, first in a study in which decreased plasma and urinary cortisol levels were reported in individuals exposed to chronic stress (Friedman et al., 1963). Patients suffering from post-traumatic stress disorder (PTSD) also exhibit low levels of cortisol (Yehuda, 2001). Disturbances in HPA activity and decreased cortisol production and/or release have been reported in

women with a history of childhood abuse (Heim et al., 2000, Heim et al., 2003), and also in patients with chronic fatigue syndrome and fibromyalgia (Crofford et al., 1994, Demitrack and Crofford, 1998, Demitrack et al., 1991). Atypical depression is also associated with hypoactivity of the HPA axis (Gold et al., 2002).

1.2.5 Cytokines, Inflammation, and Depression

Elevation of the inflammatory biomarkers is implicated in depression. Research over the last few years has consistently shown that patients with major depression exhibit evidence of activated inflammatory responses and significantly high levels of inflammatory biomarkers. For example, elevated levels of innate immune cytokines such as tumour necrosis factor-α (TNF-α), peripheral pro-inflammatory cytokines including interleukin 6 (IL-6) and downstream acute phase reactants more notably C-reactive protein (CRP) are the inflammatory biomarkers associated with major depression (Empana et al., 2005). As mentioned before, based on the macrophage theory of depression introduced by Smith, and then followed by Maes, cytokines play a role in depression. (Maes et al., 1995, Smith, 1991). The theory suggests the critical effect of elevation of pro-inflammatory cytokines that follows the acute phase reactions as a consequence in depressed individuals. The phenomenon is linked to the alteration of central nervous system (CNS) and contributes to symptoms of depressive disorder including insomnia and fatigue (Irwin and Miller, 2007).

Cytokines, in general, are proteins that are secreted by various cells and have a critical role in regulating immune response by acting as signalling molecules. They exhibit either pro- or anti-inflammatory activity. Pro-inflammatory cytokines such as IL-1, IL-6, and TNF serve to enhance the immune response, while anti-inflammatory cytokines such as

Il-4, IL-10, and IL-13 are responsible for reducing the immune response and inhibiting the synthesis of other cytokines. The cascade of cytokine production acts in a way that early cytokines stimulate the release of later cytokines. For example IL-1 induces the production of IL-2, IL-6, and TNF. The major sources of these cytokines are activated monocytes and macrophages (Dinarello, 1990). In addition, cytokines and their receptors are detected in the brain tissue. They are mainly produced by astrocytes and microglia cells and even under certain conditions by neurons (Kronfol and Remick, 2000, Schobitz et al., 1994).

Although once it was believed that due to presence of the blood-brain-barrier (BBB), the brain is protected from peripheral inflammatory activation level, the concept has been challenged due to the development in neuroimmunology research and evidence showing the presence of inflammatory responses within the CNS. In fact, cytokines may not passively cross the BBB due to their size and hydrophilic nature, at least under physiological conditions, but studies reveal that they in fact are able to penetrate the brain via specific mechanisms. The communication between cytokine and the brain can be facilitated by: active transport mechanisms, passive diffusion at circumventricular sites where the BBB is deficient, induction of specific adhesion molecules, and finally activation of peripheral afferent nerve terminals to release cytokines (Kronfol and Remick, 2000, Wichers and Maes, 2002).

Cytokine mediated inflammatory responses have been suggested to associate with behavioural alteration and development of depressive symptoms. Cytokine therapy is widely used as an effective treatment for medical conditions. However, it has been shown that for example IFN-α treatment in patients with cancer and hepatitis C as a therapeutic strategy for their condition is accompanied by neuropsychiatric side effects with

approximately half of the recipients developing clinically significant depressive symptoms (Capuron et al., 2002, Dieperink et al., 2003, Hauser et al., 2002). As revealed by animal studies, cytokine induced sickness behaviour, resembling symptoms of depression in human, is another feature observed following central or peripheral administration of pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α (Wichers and Maes, 2002).

Various mechanisms have been studied to explain the role of cytokines in the pathophysiology of mood disorders. Cytokines influence the neuroendocrine function by having an impact on the HPA axis activity. The effect has been reported to be through stimulation of CRH and ACTH expression and release as well as cortisol (Miller et al., 2009, Pariante and Miller, 2001). In addition, pro-inflammatory cytokines may generate GC resistance by directly affecting functional capacity of GR at multiple levels. Apart from induction of inert GRβ over active GRα isoform as described before, cytokines trigger GC resistance by reducing GR ligand and DNA binding capacities, inhibiting GR translocation to the nucleus and influencing GR protein-protein interactions as seen for example by activating the mitogen-activated protein kinase (MAPK) signalling pathway in cytoplasm which leads to phosphorylation of the receptor protein, thus diminishing GR transcriptional activity (Pace et al., 2007, Raison and Miller, 2003). Furthermore, in animal models exposed to either acute or chronic stress, excessive production of cytokines causes diminished neurotrophic support and neurogenesis (Ben Menachem-Zidon et al., 2008, Koo and Duman, 2008), the two features which are enhanced by cytokines within the physiological range (Goshen et al., 2007). Moreover, neuro-inflammatory activation enhances oxidative status in the CNS stimulating the production of nitric oxide, the characteristic that is observed in the neuropathophysiology of depression (Ida et al., 2008, Li et al., 2008).

In the present PhD thesis the immune system is assessed by measuring the circulatory levels of CRP and also the expression of the candidate genes associated with inflammation in order to investigate the activation of inflammatory response followed by identifying potential causes and consequences of such dysregulation.

1.2.6 Serotonin, Inflammation, and Depression

Serotonin (5-hydroxytryptamine or 5-HT) is a neurotransmitter synthesized from the amino acid tryptophan. The brain transmitter is critically implicated in a wide range of neurobehavioral phenomena such as mood, cognition, emotion, anxiety, sleep, or sexual activity (Belmaker and Agam, 2008). Indeed, as mentioned before, the monoamine hypothesis as a major theory of depression suggests the deficiency of brain serotonin as a main factor contributing to depression, and targeting the serotonin neurotransmission by medications is widely used as an effective treatment for mood and anxiety disorders, and specifically depression (Nemeroff and Owens, 2002).

One of the main factors influencing serotonergic transmission in the brain is the serotonin transporter (5-HTT) responsible for the reuptake of serotonin from the synaptic cleft into the presynaptic neurone (Breedlove et al., 2007). 5-HTT is encoded by a single gene known as SLC6A4 whose transcriptional activity is particularly modulated by SLC6A4–linked polymorphic region (5-HTTLPR), a repetitive sequence consisting of two prevalent alleles: short (S) and long (L) allele variants. The S allele of the 5-HTTLPR has been shown to reduce transcription efficiency of this gene, decrease expression of 5-HTT, and reduce serotonin reuptake from the synaptic cleft (Canli and Lesch, 2007, Collier et al., 1996). Carrying two copies of the “high transcription” L allele has been reported as a protective genotype against depression (Caspi et al., 2003). The “low transcription” SS

polymorphism is also associated with HPA axis hyperactivity, and predicts high stress-induced cortisol response (Mueller et al., 2011). Studies also suggest an interaction between the IL-6 and the 5-HTTLPR genes. Specifically, the protective effect of the "high transcription" L allele has been shown only in the presence of the "low IL-6" synthesizing (CC) genotype and, thus, low inflammation (Bull et al., 2009).

The central serotonergic system is involved in the activation and negative feedback regulation of HPA axis, and the communication between the two systems is observed during both development and adulthood (Andrews and Matthews, 2004, Lowry, 2002). Animal studies reveal that serotonin has a direct effect on hippocampal GR binding in rats (Mitchell et al., 1990) as well as the expression of GR mRNA in hippocampal neurons (Erdeljan et al., 2001). Therefore, an alteration in the serotonergic system adversely affects the regulation of HPA axis function.

Activated inflammatory responses have been suggested to induce depressive symptoms not only by directly affecting the brain but also modulating the serotonergic system suggesting a causal relationship between altered inflammation and disturbances in the serotonergic system (Dantzer et al., 2011, Wichers and Maes, 2004). The monoamine hypothesis is linked with the activation of inflammatory responses. Indeed, the availability of tryptophan synthesizing serotonin is regulated by indoleamine-2,3-dioxygenase (IDO) enzyme whose expression is altered by the activation of inflammatory cytokines (Wirleitner et al., 2003a).

Disturbances of the serotonergic system have been suggested to be associated with alteration in tryptophan metabolism. As proposed by the Lancet paper in 1969, introducing another major aetiological factor underlying development of depression,

tryptophan metabolism could be shunted away from the serotonin synthesis pathway, and instead shifted towards the formation of kynurenine metabolite (Lapin and Oxenkrug, 1969).

1.2.7 Tryptophan Metabolism, Inflammation, and Depression

Tryptophan (TRP), which is a precursor of serotonin, is an essential amino acid supplied through dietary intake of dairy products, meats, egg whites, nuts, and chocolate. The synthesis of serotonin occurs within the brain; hence TRP needs to be transported across the BBB. In fact, TRP availability to the brain relies on the peripheral determinants, as the synthesis of TRP does not occur in the brain (Badawy, 2010, Watts et al., 2012). The bioavailability of tryptophan can be affected by other competing amino acids (CAA) including leucine, isoleucine, valine, tyrosine, and phenylalanine; the association which is implicated in depression relapse in susceptible individuals (Salomon et al., 2003, Young and Leyton, 2002). Indeed, depressed patients show 20%-29% decreased in plasma tryptophan as well as 16%-36% reduced in tryptophan availability to the brain as expressed by the TRP/CAA ratio which is in fact the result of lower tryptophan rather than higher CAA (Badawy, 2010).

The decrease in tryptophan concentration in MDD patients is explained by acceleration in the degradation of this amino acid. Kynurenine pathway of tryptophan metabolism is known as one of the major mechanisms that lead to the increased tryptophan degradation (Badawy, 2013). Independently of serotonin, tryptophan metabolites also contribute to the pathogenesis of depression due to their neurotropic effect (Dantzer et al., 2011).

There are two metabolic pathways associated with TRP in humans: the kynurenine pathway and the methoxyindoles pathway. In normal conditions, about 5% of TRP is metabolized via the serotonin pathway, while the majority of around 95% is being shuttled through the kynurenine pathway in the liver. The fate of TRP depends on the comparative activities of the rate-limiting enzymes associated with each pathway (Gal and Sherman, 1980, Oxenkrug, 2007, Stone and Darlington, 2002).

As illustrated in Figure 1.3 the methoxyindoles pathway leads to serotonin biosynthesis by the catalytic activity of the enzyme tryptophan-5-hydrohylase (TPH) and an important cofactor tetrahydrobiopterin, resulting in hydroxylation of TRP and the formation of the 5-hydrohytryptophan (5-HTP) metabolite. The following decarboxylation converts 5-HTP into 5-HT. 5-HT then is either converted to a stable metabolite known as 5-hydroxyindole acetic acid (5-HIAA) catalyzed by monoamine oxidase and aldehydrogenase, or as a melatonin substrate first is converted to N-acetyl-serotonin (NAS) catalyzed by 5-HT N-acetyl transferase, and then in turn results in melatonin (5-methoxy-N-acetyltryptamine) synthesis via O-methylation. The deficiencies of the methoxyindoles pathway metabolites contribute to depressive symptoms (Gal and Sherman, 1980, Oxenkrug, 2010).

The kynurenine pathway of tryptophan metabolism is activated by the activity of two enzymes: tryptophan-2,3-dioxydase (TDO) found exclusively in the liver with high specificity for TRP, and IDO found in most tissues and organs throughout the body except the liver with less substrate specificity. Degradation of TRP by these rate-limiting enzymes induces the formation of kynurenine (KYN) as an intermediate substrate which can be converted to other catabolic products of the two distinct routes: either neuroprotective or potentially neurotoxic (Guillemin et al., 2007, Moffett and Namboodiri, 2003). In addition, KYN itself can induce IDO activation and influence TRP

availability by inhibiting its transport via BBB as observed in animal models of anxiety (Lapin, 2003).

In normal physiological conditions, TDO is the enzyme activated metabolizing TRP into KYN in the liver. However, under stress related situations including infections, oxidative stress, and especially inflammatory conditions, IDO is the first rate-limiting enzyme which is activated. It is believed that the activity of IDO enzyme is modulated by the activation of inflammatory responses and the balance between pro- and anti-inflammatory cytokines. Pro-inflammatory cytokines such as INF-γ, IL-1, IL-6, TNF-α, and CRP can enhance the activity of IDO enzyme, therefore, shifting TRP metabolism towards formation of KYN rather that serotonin synthesis (Halaris, 2013, Wichers et al., 2005). On the other hand, anti-inflammatory cytokines such as IL-4 have an inhibitory effect on IDO activity (Musselman et al., 2001). Evidence suggests that alterations in the levels of interleukins associated with KYN metabolism have been implicated in psychiatric conditions including bipolar (Kim et al., 2004) and major depressive disorders (Myint et al., 2007). Inflammatory induced IDO overstimulation is also associated with depressive symptoms as it leads to TRP depletion that in turn results in serotonin deficiency (Wichers and Maes, 2002).

As demonstrated in Figure 1.3, KYN as a substrate is competed by two different pathways. The neuroprotective pathway facilitated by KYN aminotransferase (KAT) converts KYN into kynurenic acid (KYNA) which is an endogenous N-methyl-D-aspartate (NMDA) receptor antagonist and glutamate receptor blocker, therefore potentially neuroprotective metabolite. The neurotoxic pathway leads to the transformation of KYN into 3-hydroxykynurenine (3-HK) catalysed by kynurenine 3-monooxydase (KMO) or KYN 3-hydroxylase. The subsequent step is facilitated by

Kynureninase (KYNU) to form 3-hydroxyanthranilic acid (3-HAA), the precursor of the quinolinic acid (QUIN) which is a NMDA receptor agonist and induces oxidative stress, thus neurotoxic metabolite that can potentially lead to CNS excitotoxicity (Gabbay et al., 2010, Miller et al., 2009, Oxenkrug, 2010). In the competition for conversion of KYN into its metabolites, the activity of the KMO directing the pathway through QUIN formation overcomes the KAT activity (Wichers et al., 2005). Elevation of the neurotoxins of the KYN pathway has been shown to be associated with hyperglutamatergic status in depression (Muller and Schwarz, 2008).

Depending on which cell type KYN is produced or transported, it is degraded into different metabolites. In the brain, the formation of neurotoxic 3-HK and QUIN occurs in microglia cells, while neuroprotective KYNA is mainly produced by astrocytes (Dantzer et al., 2011). Peripheral KYN is transported through BBB by a protein carrier to reach the CNS where it degrades into the neurotoxic metabolites when taken up by glia cells leading to neurodegeneration (Fukui et al., 1991). The switch of KYN neuroprotective pathway towards neurotoxic route has been proposed to be the result of inflammatory induced IDO overstimulation, the challenge that can explain the neurodegeneration hypothesis of depression (Myint and Kim, 2003). The hypothesis has been suggested to be associated with depression when microglial KYN degradation is dominant over the astrocytic metabolism (Muller and Schwarz, 2008).

Due to the effect of inflammation on tryptophan metabolism, and in turn the implication of the disturbed system in pathophysiology of depression the present PhD assesses the tryptophan metabolism pathway concentrating mainly on the kynurenine diversion of the system, and measuring the circulatory levels of the metabolites involved.

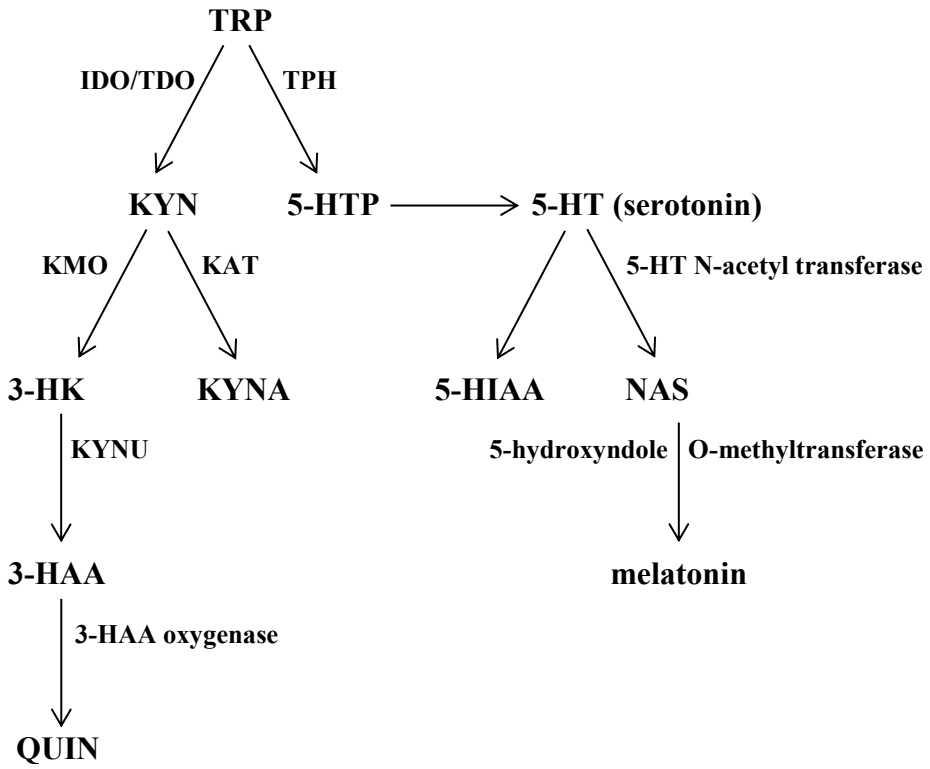

Figure 1.3 Kynurenine and methoxyindoles pathways of tryptophan metabolism

IDO, indoleamine-2,3-dioxygenase; NAS, N-acetyl serotonin; QUIN, quinolinic acid; KAT, kynurenine aminotransferase; KMO, kynurenine 3-monooxydase or kynurenine 3-hydroxylase; KYN, Kynurenine; KYNA, Kynurenic acid; KYNU, Kynureninase; TDO, tryptophan-2,3-dioxygenase; TPH, tryptophan-5-hydroxylase; TRP, tryptophan; 3-HAA, 3-hydroxyanthranilic acid; 3-HK, 3-hydroxykynurenin; 5-HIAA, 5-Hydroxyindole acetic acid; 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydrohytryptophan.

The schematic diagram above illustrates kynurenine and methoxyindoles pathways, the two metabolic pathways associated with TRP metabolism. The methoxyindoles pathway leads to serotonin biosynthesis by the catalytic activity of the enzyme TPH resulting in hydroxylation of TRP and the formation of the 5-HTP metabolite. The following decarboxylation converts 5-HTP into 5-HT. 5-HT then either is converted to a stable metabolite 5-HIAA catalyzed by monoamine oxidase and aldehydrogenase, or as a melatonin substrate first is converted to NAS catalysed by 5-HT-N-acetyl transferase, and then in turn results in melatonin synthesis via O-methylation. The kynurenine pathway is activated by the activity of two rate-limiting enzymes TDO and IDO. Degradation of TRP by these enzymes induces the formation of KYN, an intermediate substrate which is competed by two different pathways. The neuroprotective pathway facilitated by KAT enzyme converts KYN into KYNA which is an endogenous N-methyl-D-aspartate (NMDA) receptor antagonist and glutamate receptor blocker, therefore potentially neuroprotective metabolite. The neurotoxic pathway leads to the conversion of KYN into 3-HK catalysed by KMO, and then formation of 3-HAA, the precursor of the QUIN which is a NMDA receptor agonist and thus neurotoxic metabolite (Gabbay et al., 2010, Oxenkrug, 2010).

1.2.8 Antidepressants

Antidepressants are clinically used for treatment of MDD and also some other conditions such as chronic pain, anxiety and sleep disorders. The major classes of these drugs that are effectively used include the early antidepressants: tricyclic antidepressants (TCAs) (e.g. clomipramine) and monoamine oxidase inhibitors (MAOIs) (e.g. phenelzine), and the newer generation: selective serotonin reuptake inhibitors (SSRIs) (e.g. citalopram, fluoxetine, sertraline) and serotonin-norepinephrine reuptake inhibitors (SNIRs) (e.g. venlafaxine) (Nemeroff and Owens, 2002). However, the exact mechanisms of actions of these antidepressants are still under investigations.

Despite the development of various medications as antidepressants and all the investigations trying to understand the mechanisms of their action, drug resistance remains the huge challenge in treatment of depression. The concept is defined as treatment-resistant depression (TRD) when there is an inadequate response following pharmacotherapy of MDD by antidepressants. TRD leads to the condition becoming chronic, more severe and recurrent (Fava, 2003). It is relatively common affecting more than half of depressed individuals who fail to achieve remission (Greden, 2001). As reported by a systematic review, TRD is found to be associated with poorer clinical outcome especially where the multiple antidepressants are applied (Fekadu et al., 2009). Development of new strategies through identification of specific risk factors and targets seems to be a crucial step to prevent recurrence depression and if at all possible its initial development (Gotlib and Hammen, 2002).

In regards to inflammation that is involved in the pathogenesis of depression, studies on therapeutic strategies using antidepressants report that some antidepressants appear to exhibit beneficial effect through reducing levels of inflammatory biomarkers and

modulating inflammation-induced depression (Tousoulis et al., 2009). TCAs and noradrenaline reuptake inhibitors have been reported to attenuate neuro-inflammatory activation in depression by inhibiting the over expression of pro-inflammatory cytokines and also inducible nitric oxide synthase leading to reduced inflammatory reactions and oxidative stress (Lu et al., 2010, O'Sullivan et al., 2009).

Effective antidepressant treatment has been shown to resolve HPA axis dysregulation (Pariante and Miller, 2001). In fact, as reported by both *in vitro* and *in vivo* studies, these medications exert their effect directly to GR through increasing expression, promoting translocation, and enhancing function of these receptors, hence regulation of GR-mediated HPA axis activity (Carvalho et al., 2008, Carvalho et al., 2010, Pariante, 2004, Pariante and Miller, 2001). The effect of antidepressants on enhancing hippocampal neurogenesis has been also proposed by studies in animals as well as in humans (Boldrini et al., 2009, Duman, 2004, Wang et al., 2008), that are suggested to be via GR dependent mechanisms associated with modulation of GR phosphorylation and transcription (Anacker et al., 2011b).

Therefore, treatment of depression as a complex and multifaceted disorder requires more specific understanding about the underlying mechanisms involved in the pathogenesis of the disorder including cytokine induced complications, GR mediated HPA axis dysregulation, and/or neurogenesis related disturbances associated with MDD.

The present thesis analyses the effects of antidepressants *in vivo* on the various biological systems investigated, by comparing depressed patients who are drug free and patients who are on antidepressants. Moreover, it directly assesses the *in vitro* effects of

antidepressants on the GR and immune function directly on peripheral mononuclear white blood cells.

1.2.9 Polyunsaturated Fatty Acids (PUFAs)

N-3 or omega-3 polyunsaturated fatty acids (PUFAs) are derived from fish oil and considered as safe therapeutic compounds in treatment of rheumatic arthritis, Crohn's disease, cardiovascular disorders, and other systemic inflammatory conditions (Calder, 2006, Farzaneh-Far et al., 2009). In fact, omega-3 fatty acids display anti-inflammatory properties. An independent and inverse association between omega-3 levels and inflammatory biomarkers including IL-6 and CRP has been reported in patients with coronary artery disease (Farzaneh-Far et al., 2009).

Evidence suggests the relationship between omega-3 and depressive disorders indicating both protective and therapeutic effects of these agents (Su et al., 2000). Indeed, countries with higher consumption of fish oil have a lower prevalence of depression (Hibbeln, 1998). Dietary intake of omega-3 is thought to be essential since neuronal cell membrane in the CNS require high concentration of this group of fatty acids whose alteration either in terms of concentration or molecular composition can adversely affect the microstructure of cell membrane and disturbance in the signalling pathways. The mechanism has been suggested to be associated with immunological dysregulation and development of depression (Lin and Su, 2007, Logan, 2003).

MDD patients exhibit decreased levels of omega-3 in serum, red blood cells as well as fat tissue (Maes et al., 1999, Mamalakis et al., 2006, Peet et al., 1998). A therapeutic trial in MDD where omega-3 has been added to maintenance antidepressant therapy, showed a

significant improvement in symptoms of depression more specifically low mood, insomnia, and feelings of worthlessness and guilt (Nemets et al., 2002). The therapeutic effect of omega-3 has been also been shown in patients with TRD (Puri et al., 2002).

Due to potential effect of PUFAs as an anti-inflammatory and antidepressant agent, the present thesis investigates the effects of PUFAs *in vitro* on the GR and immune function in peripheral blood mononuclear cells (PBMC) to better understand the mechanisms by which these medications possibly exert their effect.

1.3 Coronary Heart Disease, Inflammation, and Depression

The association between heart disease and depression is extensively evidenced in both epidemiological and experimental studies (Davidson, 2012, Jiang et al., 2002, Kemp et al., 2003, Whooley and Wong, 2013). Inflammation is recognised to be involved in this association (Halaris, 2013, Kop and Gottdiener, 2005, Maes et al., 2011, McCaffery et al., 2006).

Heart disease refers to disorders of cardiovascular system involving heart and/or blood vessels, and CHD is the most common type in which the atherosclerotic plaques develop progressively. Atherosclerosis is in fact an inflammatory disease. Inflammation plays a central role in pathogenesis of CHD and is involved in all stages of atherosclerosis including initiation, propagation, and activation of atherosclerotic plaque leading to clot formation or thrombogenesis. Inflammation in the plaques is indeed crucial for occurrence of cardiac events such as myocardial infarction or heart attack (Hansson, 2005b, Libby, 2006).

The inflammatory response in CHD involves activation of immune cells which initiate the cytokine cascade by producing pro-inflammatory cytokines including IL-1, TNF, and INF that in turn produce IL-6 in large amounts leading to production of high levels of CRP. Indeed, elevated levels of circulating inflammatory biomarkers such as IL-6 and CRP are reported in patients with CHD that has been shown to be correlated with poor prognosis as well as increased risk of future cardiac events and sudden death (Hansson, 2005b, Pearson, 2003, Vasan et al., 2003, Willerson and Ridker, 2004).

On the other hand, excessive inflammation due to the impaired GR functioning and GC resistance has been found consistently in patients with MDD (Pace et al., 2007, Stewart et

al., 2009). However, it is postulated that GC resistance itself may occur as a result of chronic stress and prolonged exposure to inflammatory cytokines (Miller et al., 1999).

Failure of regulatory responses in the control of inflammation could be particularly important in the development of depression in CHD patients when the immune system fails to shift through anti-inflammatory induction to inhibit excessive pro-inflammatory release (Davidson, 2012). Downregulation of anti-inflammatory cytokine IL-10 and upregulation of pro-inflammatory cytokines IL-6 and TNF-α have been reported in chronic heart failure patients with depressive symptoms (Parissis et al., 2004). Another study in patients with myocardial infarction demonstrated that increased plasma IL-6 and CRP concentrations and so altered response to the anti-inflammatory properties of GCs independently correlate with depressive symptoms (Pizzi et al., 2008). However, it is still not known whether inflammation in patients with CHD is a "state" biomarker that follows the development of depression, or an "at risk" biomarker that confers vulnerability to develop depression.

A series of studies have contributed to answering this question and provided evidence that the activated inflammatory responses are indeed biomarkers of *both* vulnerability to develop depression *and* of the presence of a more severe form of depression, those less responsive to antidepressants. For example, severe treatment resistant inpatients with major depression show increased plasma levels of IL-6 in the presence of resistance of the peripheral blood GR to the anti-inflammatory action of GCs (Carvalho et al., 2008). Moreover, and consistent with the case that inflammation is indeed a vulnerability marker, Danese et al. have shown that early childhood trauma, a well-known risk factor for the future development of depression, is associated with increased levels of CRP in adulthood, even in the absence of adult depression (Danese et al., 2007). One study has

also found that the persistence of inflammation in people with a history of depression even in the absence of their depressive symptoms, again supporting the notion of inflammation as a vulnerability marker (Kling et al., 2007).

1.4 Aims and Hypotheses of the Study

The INFLAME-BEAT project is the first clinical study directly investigating inflammatory-induced depression in CHD patients. The goal of the study is to directly test the hypothesis that inflammation in patients with CHD is a state biomarker of depression. Therefore, the aim of the present thesis, as part of the INFLAME-BEAT study, is the investigation of the pathophysiological mechanisms underlying the inflammatory activation in CHD patients with and without depression. Indeed, the present project evaluates inflammatory response in patients with CHD and with current depression and compares to those without depression at baseline. Considering the presence of GC resistance in patients with depression and the idea that chronic inflammation may induce depression through alteration in GR functional properties, this PhD thesis aims to investigate also two important biological elements in the pathogenesis of major depression in CHD patients; namely the HPA axis activity and GR function. Furthermore, this PhD project intends to explore the adverse consequences of inflammation and depression in CHD patients by examining the kynurenine pathway of tryptophan metabolism in this population.

The hypotheses that the present thesis is trying to test are as follows:

As compared to CHD patients without depression, CHD individuals with depression:

1) Have higher circulating levels of CRP as a clinical inflammatory marker.
2) Have higher expression levels of cytokines as markers of inflammation.
3) Display lower expression of inflammatory related genes in those who are on antidepressant treatment.
4) Show HPA axis alteration demonstrating higher levels of cortisol both in plasma and saliva.

5) Exhibit GR resistance displaying lower expression and sensitivity of GR.
6) Show improvement in GR sensitivity following *in vitro* effect of antidepressants and omega-3 fatty acids.
7) Demonstrate disturbances in tryptophan metabolism pathway showing activation of IDO enzyme and production of neurotoxic metabolites of the kynurenine pathway.
8) Have higher levels of circulating VEGF.

Comprehensive understanding of the cause(s) and targeting directly the specific mechanisms involved in the pathogenesis of depression as a complex multifactorial disorder are essential to achieve effective treatment of depression, probably through multipotential therapy. In addition, uncovering the mechanisms underlying the inflammatory activation in patients with heart disease will help develop strategies of prevention and identifying treatment targets to improve mental health in patients with physical illnesses.

Chapter 2 METHOD

This research has been approved by the National Research Ethics Service, East Kent Local Research Ethics committee (reference number 09/H1103/19); The Institution Review Boards at the Institute of Psychiatry, King's College London (reference numbers 07/H0809/38 and 322/2003); and the University of Roehampton Ethics Committee (reference number BHS 10/025).

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2.1 Study Design, Subjects and Sample Collection

This project, INFLAME-BEAT, was conducted in collaboration with an existing and on-going study called UP-BEAT led by Prof. Andre Tylee (Institute of Psychiatry, IOP; KCL) examining patients with CHD (recruited through primary care services in South-East London) and evaluating psychosocial, but not biological, predictors of depression in CHD. The purpose of the INFLAME-BEAT study was to evaluate underlying biological mechanisms involved in development of depression in CHD patients. INFLAME-BEAT utilized a four-year prospective cohort design while the biological analysis was conducted only once at baseline. The present PhD is a cross-sectional study where CHD patients without depression were compared to CHD patients with depression for their circulatory inflammatory biomarkers, HPA axis assessments, GR sensitivity, expression of genes associated with inflammation and GR as well as kynurenine pathway of tryptophan metabolism.

UP-BEAT participants comprised 800 patients who were registered on the CHD Quality and Outcomes Framework (QOF) from participating practices. All volunteers who scored positive in the patient health questionnaire-2 (PHQ2) scale for depression had baseline psychiatric evaluation of mental state and previous psychiatric history assessed by UP-BEAT group using the Clinical Interview Schedule-Revised (CIS-R) (Lewis et al., 1992). As part of the UP-BEAT study, CHD patients were clinically assessed once at baseline and then followed up every 6 months for 4 years for development of depression.

INFLAME-BEAT subjects were recruited from UP-BEAT study participants who were interested to take part in other related studies and gave permission to be contacted by associated studies. The volunteers randomly were contacted and invited to participate in the INFLAME-BEAT project (see appendix-A/-B). The priori exclusion criteria included

patients taking corticosteroid medications such as prednisolone. In addition, patients with medical co-morbidity including asthma, cancer, and arthritis as well as those suffering from acute illnesses or infections were excluded. Furthermore, patients with other psychiatric conditions according to the CIS-R assessment were also excluded. The conditions included panic, obsessive–compulsive disorder (OCD), phobias (social phobia, specific phobias, agoraphobia), generalised anxiety disorder, and mixed depression anxiety disorder. The flowchart below demonstrates recruitment procedure carried out by this study.

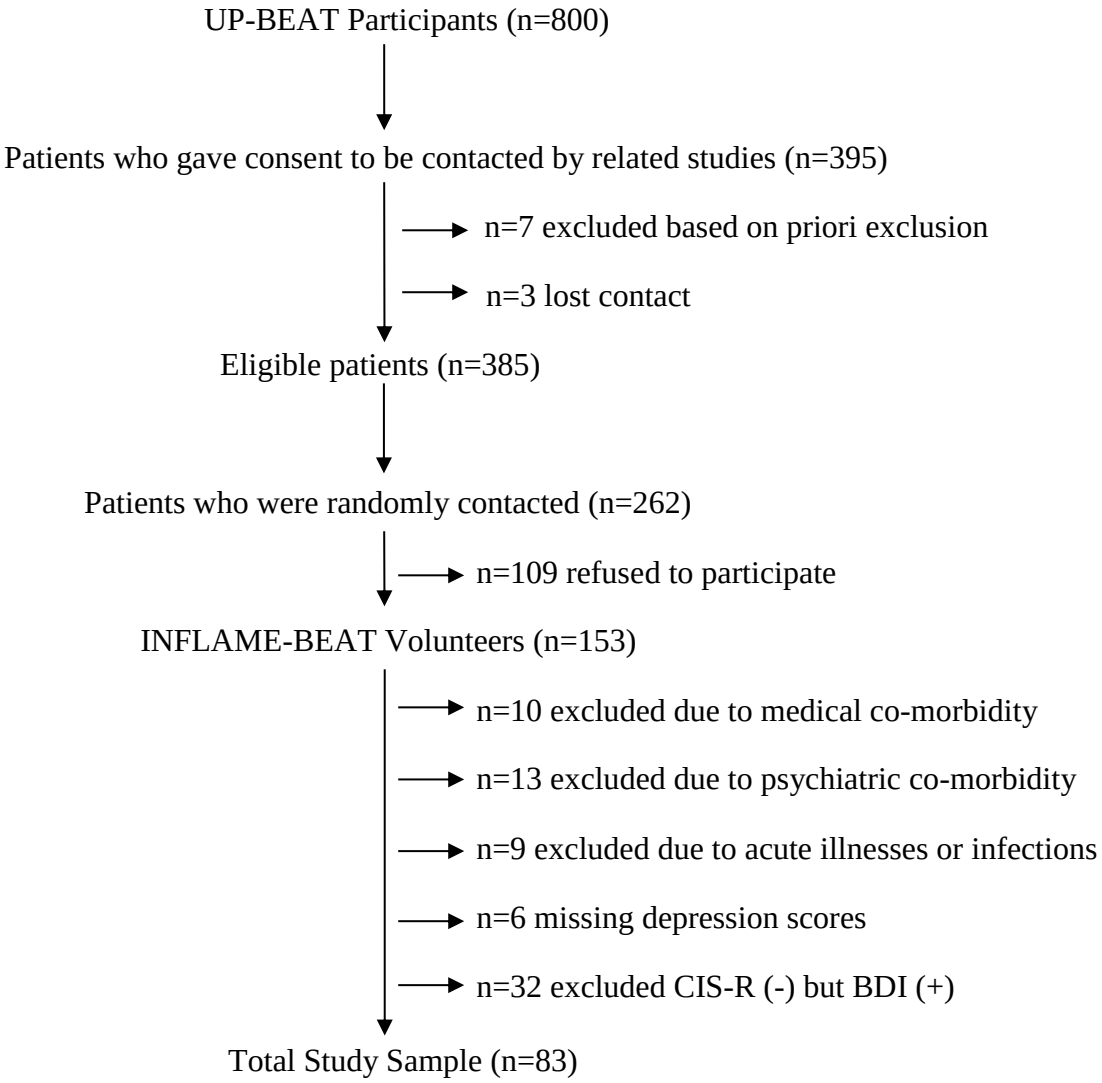


Figure 2.1 Flowchart of recruitment procedure

2.1.1 Depression Assessments

CHD patients with and without depression were recruited. The subjects were categorized into the two groups: group 1 were patients with symptomatic CHD and without current depression; and group 2 were those with CHD and currently depressed. The depression status was defined based on the CIS-R assessment (Lewis et al., 1992) for diagnosis of depression obtained from UP-BEAT database; as well as the presence of depressive symptoms that were quantified using the Beck depression inventory (BDI) score (Beck et al., 1961) obtained at the time of the biological sample collection. The cut-off BDI scores ≥10 was used indicating the presence of depressive symptoms (Beck et al., 1988), that has been used previously by various studies (Bull et al., 2009, Meurs et al., 2012). Therefore, CHD patients who were both CIS-R and BDI positive were categorized as CHD depressed (CHD-D), and CHD patients who were both CIS-R and BDI negative were categorized as CHD non depressed (CHD).

Apart from the PBMC cell culture (section 2.5.3); all the experiments described here in the following sections were preformed blind to the subjects' depression status. The advantage of using frozen PBMC cells was that *in vitro* assessment of GR function could be performed in parallel for depressed and non-depressed CHD individuals.

2.1.2 Blood Samples

On attendance at Kings College Hospital London, patients were interviewed, and asked to complete the BDI (see appendix-E). The information in regards to their medication was recorded, and blood pressure, heart rate, height, and weight were measured (see appendix-D). Informed consent was obtained from each participant (see appendix-C) followed by collecting peripheral venous blood (simple venepuncture): 4 sodium-heparin containing

tubes for collecting plasma and isolation of PBMC, a clot activator containing tube for collecting serum, and a PAXgene tube for isolation of RNA. The blood samples were collected between 8:30 am to 10:30 am.

In addition, 12 healthy controls were recruited (at the University of Roehampton) without any history of heart disease and depression. Heparinised whole blood was obtained from healthy donors to be used for optimizing the PBMC cell culture protocol.

2.1.3 Saliva Samples

Saliva samples were collected from participants to measure their salivary cortisol during the day according to a previously published procedure (Mondelli et al., 2010). Participants were issued with the materials and instruction for collecting the saliva samples at 6 time points in a single day (see appendix-F/-G/-H). The self-collection of samples was carried out at home, and patients agreed to follow the procedure accurately. Patients were required to wake up before 10 am on the day, and obtain saliva samples immediately upon awakening (0 minutes) while still in bed, and then at 15, 30, and 60 minutes post awakening, and again at 2 pm and bedtime at around 10 pm. To obtain saliva samples, subjects were issued 6 labelled salivette sampling devices (Sarstedt, Leicester, UK). They were instructed to remove the cotton roll from inner tubing, place it in their mouth, and chew on it for approximately 2 minutes to ensure a sufficient amount of saliva was absorbed for analysis. The cotton roll was then placed back into the inner tube, and after replacing the lid, the sample tube was placed in the fridge. The same procedure was repeated for each time point of sampling. Participants were required not to have breakfast and not to brush their teeth, or eat or drink for the first hour of awakening, and also in the 30 minutes before 2 pm and bedtime sampling. This was asked in order to

avoid bleeding in oral cavity or food-stimulated cortisol rises that could result in false increase in cortisol levels (Mondelli et al., 2010). In addition, participants were instructed to specify the exact time of each sampling and to provide information with regards to whether they accidentally had anything to eat or drink, and any difficult or tense situation, unpleasant thoughts or any kind of pain before taking the sample (see appendix-H). Individuals who described problems during sample collection in the self-recorded questionnaire provided, or who did not respect the time-intervals required, were taken out of the analysis (3 participants were excluded due to protocol deviation). Furthermore, participants were asked to fill up BDI questionnaire (see appendix-E) on the day of sampling.

Upon completion of collecting six saliva samples in a single day and keeping them refrigerated overnight, patients were asked to return all sample tubes on the following morning together with the completed information sheet and BDI questionnaire in a pre-paid padded envelope provided. Once received, the samples were stored in -20 ºC freezer until assayed.

The methods used for various measurements are described in the subsequent sections. The numbers of patients varied between the studies due to lack of availability or insufficient samples when performing different experiments. It should be also noted that not all the patients who provided blood samples also agreed to provide saliva sample. In the other hand, some patients were only interested to donate saliva samples and not blood. In addition, for the GR function assessment, PBMC were not available for all the patients who were investigated for their gene expression analysis. Therefore, each experiment was performed on a subset of the study population based on the availability of the sample for that specific experiment.

2.2 Peripheral Cortisol and CRP Measurements

The blood samples collected in sodium-heparin and clot activator containing tubes were centrifuged to separate plasma and serum, respectively. The samples were stored in -80 ºC freezer until assayed. For analysis, the plasma and serum samples were sent to biochemistry laboratory, KingsPath, at King's College Hospital where the measurements of the levels of CRP in serum and cortisol in plasma took place using commercially available ELISA kits and following the recommended procedure. The report of the method used was sent to me as described in the following paragraphs with a very few editing changes.

The levels of peripheral CRP were determined performing the Cormay high sensitivity CRP (HsCRP) assay (P.Z. Cormay, Lublin, Poland) (Peters et al., 2012). Briefly, the assay employed an anti-CRP antibody which had been sensitized to latex particles. Coating the surface of latex particles by antibody resulted in the formation of milky appearing sensitized latex. CRP within the serum samples then reacted with this antibody, forming visible agglutination. The purpose of using latex particles was magnifying the antigen-antibody complex. The amount of CRP within the samples was proportional to the degree of agglutination detected as a reduction in the intensity of transmitted light at 572 nm. The assay was analysed on the Siemens Advia 2400. The minimum detectable concentration of hsCRP was 0.01 mg/dL. Inter and intra-assay co-efficient of variations were <10%.

Cortisol reagents were supplied by Siemens Healthcare Diagnostics Ltd, Camberley, Surrey, UK (Burrage et al., 2009). The plasma cortisol measurements, samples were first incubated with a 'Lite reagent' which was cortisol labelled with acridinium ester; and a 'solid-phase reagent' which was an anti-cortisol antibody bound to paramagnetic particles

(PMP). The incubation facilitates competing labelled cortisol with cortisol in the plasma samples for the PMP-bound antibody binding sites. Next, a magnetic field was applied to the reaction mixture causing the solid-phase PMP containing the bound labelled cortisol to be held at the side of the reaction cuvette while the liquid phase was aspirated. The cuvette contents were washed with deionised water which was then aspirated, again with the magnetic field applied. Acid reagent containing hydrogen peroxide was then added to the cuvette to begin the light-emission reaction with the acridinium ester. The cuvette was then moved to the luminometer at which point base reagent was added to enhance the light reaction. The light intensity was measured immediately and converted to 'relative light units' (RLU). The RLU value indicated an inverse relationship with cortisol concentration. Therefore, the more labelled cortisol was bound, the less patient cortisol would be bound. The RLU value was compared with those of the analyte master curve to obtain the analyte concentration via the relevant curve-fit algorithm. The minimum detectable concentration was 30 nmol/L. Inter and intra-assay co-efficient of variations were <10%.

2.3 Measurement of Salivary Cortisol

Salivary cortisol levels were measured using commercially available high sensitivity salivary cortisol enzyme immunoassay kit (Product code: 1-3002, Salimetrics) (Mondelli et al., 2010). The recommended procedure was followed. Salivettes containing saliva samples were taken out of the freezer and left in room temperature 1 hour before the assay. After thawing the samples completely, they were centrifuged at 3000 rpm for 15 minutes at room temperature. Centrifuging was performed to obtain a clear supernatant of low viscosity in order to avoid falsely elevated results due to mucin and other particulate matter which might interfere with the antibody binding. The procedure was carried out by adding 25 µl of standards, controls and saliva samples into appropriate wells of a microtitre plate coated with anti-cortisol monoclonal antibodies. All the samples were assayed in duplicate. 200 µl of diluted enzyme conjugate solution (1:1600) was then added to each well. Therefore, cortisol linked to horseradish peroxidase (HRP) competed with cortisol in the standards and unknowns for the antibody binding sites. After 2 hours incubation, the plate was washed 4 times using a plate washer to wash away unbound components. 200 µl tetramethylbenzidine (TMB) solution was then added to each well followed by mixing on a plate rotor for 5 minutes and incubating the plate in the dark at room temperature for an additional 25 minutes. Therefore, bound cortisol peroxidase was measured by the reaction of the peroxidase enzyme on the substrate TMB. The reaction which produced a blue colour was then stopped by adding 50 µl stop solution of sulfuric acid turning the colour to yellow. Optical density was read on Beckman Coulter DTX 880 plate reader, with Multimode Detection Software 2.0.012, at 450nm with correction at 620nm. By measuring the intensity of colour, the amount of cortisol peroxidase was detected that was inversely proportional to the amount of cortisol present in the samples; so the darker the colour, the lower concentration of cortisol. SoftMax Pro 4.8 software was used to calculate the cortisol values, following a 4-parameter fit. The analytical

sensitivity was set to 0.33 nmol/l. Inter and intra-assay co-efficients of variations ranged from 8% to 11% and 6% to 10%, respectively. Salivary cortisol measurements were conducted by Dr Patricia Zunszain, senior laboratory coordinator, at Stress, Psychiatry and Immunology laboratory (SPI-Lab), KCL.

To investigate the responsiveness of the HPA axis, the repeated measures of salivary cortisol was used to calculate the area under the curve (AUC) of the cortisol awakening response (CAR) and diurnal cortisol with respect to the increase (AUC_I) as well as with respect to the ground (AUC_G). The formulas for the calculations of the AUC were derived from the trapezoidal formula introduced by Pruessner and colleagues (Pruessner et al., 2003).

Repeated measures with variable time between measurements:

AUC for CAR:

$AUC_G = (A+B)/2 \times t_1 + (B+C)/2 \times t_2 + (C+D)/2 \times t_3$

$AUC_I = [(A+B)/2 \times t_1 + (B+C)/2 \times t_2 + (C+D)/2 \times t_3)] - [A \times (t_1 + t_2 + t_3)]$

$AUC_I = AUC_G - [A \times (t_1 + t_2 + t_3)]$

AUC for diurnal cortisol:

$AUC_G = (A+E)/2 \times t_4 + (E+F)/2 \times t_5$

$AUC_I = [(A+E)/2 \times t_4 + (E+F)/2 \times t_5] - [A \times (t_4 + t_5)]$

$AUC_I = AUC_G - [A \times (t_4 + t_5)]$

A: cortisol measure at awakening
B: cortisol measure at 15 minutes
C: cortisol measure at 30 minutes
D: cortisol measure at 60 minutes
E: cortisol measure at 2 pm
F: cortisol measure at bedtime (10 pm)

t_1: time between A & B 15 minutes

t_2: time between B & C 15 minutes

t_3: time between C & D 30 minutes

t_4: time between A & E

t_5: time between E & F

Prior to performing the calculations, the data for each measure has been double checked against the specific time point required for collecting the saliva samples. There were also missing data due to unavailability of sample, insufficient saliva sample, or not collecting sample in the specific time required. Individuals who had missing data at awakening point were excluded (2 patients). For those who had missing data at 15 min or 30 min, the average of the two points before and after the missing value was used. For missing data for 15 min, the average of awakening and 30 min was used, and for 30 min, the average of 15 min and 60 min (see appendix-I). 14 patients (7 in each group), with missing data at either 15 min or 30 min, had values interpolated using this procedure.

2.4 Gene Expression Analysis

Gene expression experiment was conducted by Dr Izabela Barbosa at SPI-Lab, KCL. The following sections describe the method used.

2.4.1 RNA Isolation from PAXgene and cDNA Synthesis

Blood samples for gene expression of IL6, NFkB, TNF-α, IL1β, and GR Total were collected using PAXgene Tubes (Qiagen). After blood samples were withdrawn, PAXgene tubes were inverted few times and then kept for 3 hours at room temperature in order to achieve complete lysis of blood cells. The tubes were placed first at -20ºC for 48 hours and then stored at -80ºC until they were processed.

Isolation of RNA was performed using the PAXgene Blood RNA Kit (Qiagen) according to manufacturer´s instructions. In brief, samples were defrosted by incubating them for the minimum of 3 hours prior to processing. The blood was pelleted, washed, and then re-suspended with a lysis buffer and proteinase K to digest cellular proteins. Samples were then passed through PAXgene Shredder spin columns to homogenise the lysate and filter out cell debris. The supernatant of the flow-through was then passed through the PAXgene RNA spin column where the silica membrane selectively binds to the RNA. After several wash steps the RNA is eluted and heat-denatured (see appendix-M). The RNA quality and quantity were assessed by evaluation of the A260/280 and A260/230 ratios using a Nanodrop spectrometer (NanoDrop Technologies, USA). Samples were kept frozen at -80 ºC until further use. One microgram of total RNA was used for cDNA synthesis and for subsequent gene expression analysis in quantitative Real Time PCR (qPCR).

2.4.2 Quantitative Real Time PCR (qPCR)

Gene expression analysis of candidate genes involved in GR function (GR total) and inflammatory responses (IL-6, NFkB, TNF-α, and IL1β) was conducted via qPCR. qPCR was performed using HOT FIREPol EvaGreen qPCR (Solis BioDyne, Tartu, Estonia) according to the SYBR Green method. For each target primer set a validation experiment was performed to demonstrate that PCR efficiencies were within the range of 90-100% and approximately equal to the efficiencies of the reference genes; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), beta-actin (ACTB), and beta-2-microglobulin (B2M). Briefly, after an initial heating step at 95 ºC for 15 min to activate the polymerase, 45 PCR cycles were performed. Each cycle consisted of a denaturation step at 95 ºC for 30 sec, an annealing step at 60 ºC for 30 sec and an elongation step at 72 ºC for 30 sec. Each sample was assayed in duplicate and each target gene (IL6, NFkB, TNF-α, IL1β, and GR Total) was normalized to the geometric mean of the expression of the three housekeeping genes as reference. Pfaffl method (Pfaffl, 2001) was used to quantify the relative expression levels of each target gene according to the following equation:

$$R = \frac{(E_{target})^{\Delta CP_{target}\,(control-sample)}}{(E_{ref})^{\Delta CP_{ref}\,(control-sample)}}$$

As it is shown in the equation the result is expressed as Relative Expression Ratio (R) representing the ratio of a target gene which is expressed in a sample of interest versus a control (in this study CHD depressed versus CHD non-depressed) in comparison to a reference gene. In the equation, E represents the real time PCR efficiency of target/reference gene transcript, and ΔCP is the CP (or Ct) values of the sample and control.

This method has been previously used (Anacker et al., 2011b, Cattaneo et al., 2013). The primer sequences are listed in the Table 2.1.

Table 2.1 Primer sequences of the primers used for gene expression experiment.

Primer name	Primer sequence
Glucocorticoid receptor (GR) forward	5'-AACTCTGCCTGGTGTGCTCT-3'
Glucocorticoid receptor (GR) reverse	5'-GCTGTCCTTCCACTGCTCTT-3
Tumor necrosis factor-alpha (TNF-α) forward	5'-CTGCTGCACTTTGGAGTGAT-3'
Tumor necrosis factor-alpha (TNF-α) reverse	5'-CAGCTTGAGGGTTTGCTACA-3'
Nuclear Factor-kappaBeta (NF-kB) forward	5'-GTGAGGTGGGATCTGCACT-3'
Nuclear Factor-kappaBeta (NF-kB) reverse	5'-CTCTGTCATTCGTGCTTCCA-3'
Interleukin-6 (IL-6) forward	5'-AAGCCTGACCACGCTTTCTA-3'
Interleukin-6 (IL-6) reverse	5'-GCTCCCTGGTTTCTCTTCCT-3'
Interleukin1-β (IL-1β) forward	5'-ACGATGCACCTGTACGATCA-3'
Interleukin1-β (IL-1β) reverse	5'-GAACACCACTTGTTGCTCCA-3'
glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward	5'-CAATGACCCCTTCATTGACC-3'
glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reverse	5'-TGGAAGATGGTGATGGGATT-3'
beta-actin (ACTB) forward	5'-CTCTTCCAGCCTTCCTTCCT-3'
beta-actin (ACTB) reverse	5'-AGCACTGTGTTGGCGTACAG-3'
beta-2-microglobulin (B2M) forward	5'-ACTGAATTCACCCCCACTGA-3'
beta-2-microglobulin (B2M) reverse	5'-CCTCCATGATGCTGCCTTACA-3'

2.5 GR Function Evaluation

GR function was measured directly on previously isolated and stored peripheral blood mononuclear cells (PBMC) by thawing and culturing them using "glucocorticoid inhibition of lipopolysaccharide (LPS)-stimulated IL-6 levels" technique. In this method, the cells were first stimulated by LPS which is highly immunogenic causing the cells to produce IL-6. Next, dexamethasone (DEX), synthetic glucocorticoid, was used at different concentrations to inhibit the production of IL-6 facilitated by GR. Therefore, as the higher DEX concentration, the less IL-6 is expected to be produced. However, in case of GR impairment, there would not be a decrease in the production of IL-6. IL-6 levels were measured using IL-6 ELISA assay, and so GC mediated-GR function could be evaluated.

2.5.1 Isolation and Cryopreservation of Human Peripheral Blood Mononuclear Cells (PBMC)

Sodium-heparinized blood was diluted 1:1 with phosphate buffered saline (PBS). PBMC were isolated by Ficoll-Paque density gradient centrifugation, as per previous publications (Knijff et al., 2007, Sarkar et al., 2003). The procedure was carried out by layering diluted blood on top of Ficoll (2:1 e.g. 30 ml diluted blood on 15 ml of Ficoll) in a 50 ml polypropylene tube. The tube was then centrifuged at 1000g for 15 min at room temperature, without the break. Using a sterile pastures pipette the buffy coat layer containing PBMC was collected into a new 50 ml polypropylene conical tube. PBMC re-suspended in PBS to the total volume of 50 ml, and centrifuged at 800 g for 10 min at room temperature. The supernatant was discarded and PBMC rich pellet was re-suspended in a cryovial in 1 ml cold cell culture fluid 1 (CF1) medium consisted of 10%

FCS (fetal calf serum), and 1% PEN-STREP (Penicillin-Streptomycin), and 90% RPMI 1640 with 25 mM Hepes and Ultraglutamin-1 medium. After labeling, the cryovials containing cell suspension were kept on ice. PBMC were counted using hemacytometer and trypan blue exclusion method (please see the next section 2.5.2 for detailed explanation).

Freshly prepared cell culture fluid 2 (CF2) containing 80% CF1 and 20% DMSO (dimethylsulfoxide, Me2SO) were then added 1:1 slowly in droplets to the cell suspension. The cells were distributed in 1 ml aliquots in precooled cryovials ready for storage using slow temperature-lowering method by incubating them in an isopropyl alcohol bath in Cryo 1ºC freezing container (Mr. Frosty polyethylene vial holder). The cryovials in the freezing container were placed into the -80 ºC freezer overnight and then transferred into liquid nitrogen for long term storage until future use (see the appendix-J for the protocol and step by step procedure).

2.5.2 Thawing of Cryopreserved Cells

The cryovials containing PBMC were taken out from liquid nitrogen. The cryovials were thawed immediately in a 37 ºC water bath. No more than 2 cryovials were thawed at the same time. To dilute the PBMC and wash out the DMSO, while still a few crystals of ice were visible, the cells were transferred to a 15 mL corning tube with 10 mL of warmed CF1 inside. The tubes were centrifuged at 800 g for 5 min at room temperature. The supernatants were discarded and the cells were re-suspended in 1 ml of CF1.

Recovery and viability of PBMC were examined by trypan blue exclusion technique. Cryopreserved and thawed cells contained greater than 95% viable recovered cells.

PBMC were counted using hemacytometer. A cover glass was placed in the center of the hemacytometer. Re-suspended cells were stained using 0.4% trypan blue solution. One hemacytometer chamber was filled with 10 µl of the cell and dye solution. Under the microscope cells were counted in the four corners of 4x4 areas. To determine PBMC count the mean was used multiplied by dilution factor (e.g. 10 µl PBMC solution + 40 µl PBS + 50 µl 0.4% trypan blue solution; dilution factor = 10). The cell count were recorded as number of cells in million per ml. The amount of 100K cells required for each well in a total volume of 200 µl was then calculated.

$x \times 0.2$ (ml) = 100.000 (cells/well)

$x = 500.000 = 50 \times 10^4$ (cells/ml)

$C1 \times V1 = C2 \times V2$

Number of cells counted (cells/ml) × x (ml) = 50×10^4 (cells/ml) × 0.2 (ml)

x (ml) = [50×10^4 (cells/ml) × 0.2 (ml)] / [Number of cells counted (cells/ml)]

2.5.3 Cell Culture

PBMC were cultured in a 96-well round-bottomed tissue culture plate (Falcon, 3077) at a concentration of 50×10^4 cells/ml in CF1. The following factors were either present or not in the culture according to the template below representing the culture plate designed for each PBMC sample: LPS of Escherichia Coli (E.coli) in a concentration of 1 ng/ml to stimulate IL-6 production; DEX in concentrations of 10^{-6}, 10^{-7}, $3x10^{-8}$, 10^{-8}, 10^{-9} M to inhibit the production of LPS-induced IL-6; and clomipramine (CMI), citalopram (CIT), and omega-3 fatty acid, eicosapentaenoic acid (EPA), in a concentration of 10^{-5} M to investigate the effect of these drugs on GR function. Drug concentrations were used based

on previously published studies such as (Carvalho et al., 2010, Carvalho et al., 2008) for antidepressants and (Lu et al., 2010) for EPA. Ethanol (ETOH) also was added to the control wells at a concentration of 10^{-5} M, since DEX, CIT, and EPA were diluted in ETOH. Each condition was assayed in duplicate.

The cells culture template is presented below (Table 2.2 on page 76). The template illustrates the conditions in which the cells were treated. The columns 1 and 8 contained the PBMC cultured in CF1 and without stimulation. In all the other wells the same amounts of LPS were also added, so the cultured PBMC were induced for the production of IL-6. As shown in the template, in the columns 4 to 7 and 10 to 12, DEX was added from higher to lower concentrations (10^{-6}, 10^{-7}, $3x10^{-8}$, 10^{-8}, 10^{-9} M) to obtain the dose response curve. The rows represent the conditions in which the drugs were also added (CMI in C & D; CIT in E & F; and EPA in G & H rows).

PBMC were then incubated for 24 h at 37 ºC, under 5% CO and humidified air. After 24 hrs, the plate was centrifuged at 1000 g for 10 min at 4 ºC. The supernatant from each well were harvested and transferred to a labelled Eppendorf. The culture supernatants were then stored at -20 ºC until they were assayed for measurements of IL-6 levels in the following day. The samples were then transferred to -80 ºC for long term storage.

Drugs and reagents: LPS, DEX, CMI, CIT, and EPA were purchased from Sigma. LPS was dissolved in PBS (1 mg/ml) and aliquots stored at -20 ºC. DEX was dissolved in ETOH freshly prepared before use. CMI was dissolved in PBS freshly prepared before use. CIT and EPA were dissolved in ETOH (10 mg/ml) and aliquots stored at -20 ºC. All the subsequent dilutions for the required concentrations were made in CF1 fresh before use (see the appendix-K for the protocol and step by step procedure).

Table 2.2 Cell culture template

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Unst	LPS	LPS $DEX10^{-6}$	LPS $DEX10^{-7}$	LPS $DEX3x10^{-8}$	LPS $DEX10^{-8}$	LPS $DEX10^{-9}$	Unst ETOH	LPS ETOH	LPS $DEX10^{-6}$ ETOH	LPS $DEX10^{-7}$ ETOH	LPS $DEX3x10^{-8}$ ETOH
B	Unst	LPS	LPS $DEX10^{-6}$	LPS $DEX10^{-7}$	LPS $DEX3x10^{-8}$	LPS $DEX10^{-8}$	LPS $DEX10^{-9}$	Unst ETOH	LPS ETOH	LPS $DEX10^{-6}$ ETOH	LPS $DEX10^{-7}$ ETOH	LPS $DEX3x10^{-8}$ ETOH
C	Unst CMI	LPS CMI	LPS $DEX10^{-6}$ CMI	LPS $DEX\ 10^{-7}$ CMI	LPS $DEX3x10^{-8}$ CMI	LPS $DEX10^{-8}$ CMI	LPS $DEX10^{-9}$ CMI				LPS $DEX10^{-8}$ ETOH	LPS $DEX10^{-9}$ ETOH
D	Unst CMI	LPS CMI	LPS $DEX10^{-6}$ CMI	LPS $DEX10^{-7}$ CMI	LPS $DEX3x10^{-8}$ CMI	LPS $DEX10^{-8}$ CMI	LPS $DEX10^{-9}$ CMI				LPS $DEX10^{-8}$ ETOH	LPS $DEX10^{-9}$ ETOH
E	Unst CIT	LPS CIT	LPS $DEX10^{-6}$ CIT	LPS $DEX10^{-7}$ CIT	LPS $DEX3x10^{-8}$ CIT	LPS $DEX10^{-8}$ CIT	LPS $DEX10^{-9}$ CIT					
F	Unst CIT	LPS CIT	LPS $DEX10^{-6}$ CIT	LPS $DEX10^{-7}$ CIT	LPS $DEX3x10^{-8}$ CIT	LPS $DEX10^{-8}$ CIT	LPS $DEX10^{-9}$ CIT					
G	Unst EPA	LPS EPA	LPS $DEX10^{-6}$ EPA	LPS $DEX10^{-7}$ EPA	LPS $DEX3x10^{-8}$ EPA	LPS $DEX10^{-8}$ EPA	LPS $DEX10^{-9}$ EPA					
H	Unst EPA	LPS EPA	LPS $DEX10^{-6}$ EPA	LPS $DEX10^{-7}$ EPA	LPS $DEX3x10^{-8}$ EPA	LPS $DEX10^{-8}$ EPA	LPS $DEX10^{-9}$ EPA					

Unst, unstimulated; LPS, lipopolysaccharide; DEX, Dexamethasone; CMI, clomipramine; CIT, citalopram; EPA, eicosapentaenoic acid; ETOH, ethanol

2.5.4 Cytokine Assay

The levels of pro-inflammatory cytokine IL-6 in supernatants of the PBMC cultures were measured via IL-6 specific enzyme-linked immunosorbant assay (ELISA) as per previously published work with slight modifications (Knijff et al., 2007). A 96-well ELISA plate (Costar 3590) was coated overnight at 4 ºC adding 100 µl diluted (1:1000) IL-6 specific capture antibody (Invitrogen: Ms mAb Anti-Hu IL-6) to each well. After overnight incubation, the wells were then emptied and tapped on a paper filter. Next, 300 µl block buffer, PBS containing 0.5% BSA (bovine serum albumin, Sigma) solution, was added to each well, and the plate was incubated for 2 h in room temperature. After blocking step, the plate was washed 4 times using the plate washer machine and freshly prepared wash buffer (MilliQ water containing 0.9% Nacl and 0.05% Tween20 solution). The supernatant samples were diluted to a measurable solution (1:2, 1:100, and 1:50 for Unst, LPS, and DEX, respectively) in working buffer (PBS containing 0.5% BSA). The standard solutions of IL-6 (R&D: recombinant human IL-6) were prepared in serial dilution (500, 250, 125, 62.5, 31.25, 15.63, 7.8, 3.9, 1.95, 0 ng/ml) for the standard curve. 100 µl of diluted samples and standard solutions were aliquoted in duplicate in the appropriate wells. 50 µl diluted (1:500) IL-6 detection antibody (Invitrogen: Ms mAb Anti-Hu IL-6 Biotin) were thereafter added to each well followed by incubation for 2 h on a plate shaker at room temperature. After another washing step, 100 µl diluted (1:15000) streptavidine poly HRP (Thermo scientific) was added to enhance the reaction, and the plate was incubated for 30 min under continuous shaking at room temperature. The plate was then washed 5 times. The reaction was developed in dark after adding 100 µl of TMB substrate (Thermo Scientific). The reaction was then stopped by adding 100 µl 1 M H_2SO_4 when the colour of Blank and the standard solution in the lowest concentration started to turn slightly blue (approximately after 3-5 min). The absorbance

at 450/620 nm was measured using ELISA reader (MULTISKANEX, Thermo Electron Corporation) (see the appendix-L for protocol and step by step procedure).

The standard curve was used to calculate concentrations of IL-6 in samples of supernatants using GraphPad Prism Software version 4.03 for windows, Inc., San Diego, California, USA. Inter- and intra-assay variations were <10%. The analytical sensitivity was set to 2 ng/mL. The values obtained from the levels of IL-6 of the unstimulated cells were deducted from the LPS-stimulated IL-6 levels for each condition. The calculation of percentage inhibition for each concentration of dexamethasone was as follows:

$$\text{IL-6 levels \% (DEX only)} = \frac{\text{Mean IL-6 levels (LPS-stimulated with DEX)}}{\text{Mean IL-6 levels (LPS-stimulated without DEX)}} \times 100$$

Glucocorticoid suppression was calculated by normalizing the data to LPS-stimulated IL-6 levels in the absence of dexamethasone expressed by 100% (Carvalho et al., 2010). IC50-DEX [M] representing the DEX concentration that would give 50% inhibition effect on IL-6 production was derived from the DEX dose response curve using GraphPad Prism software.

The procedure of *in vitro* evaluation of GR function directly on PBMC is summarized and illustrated in the figure below.

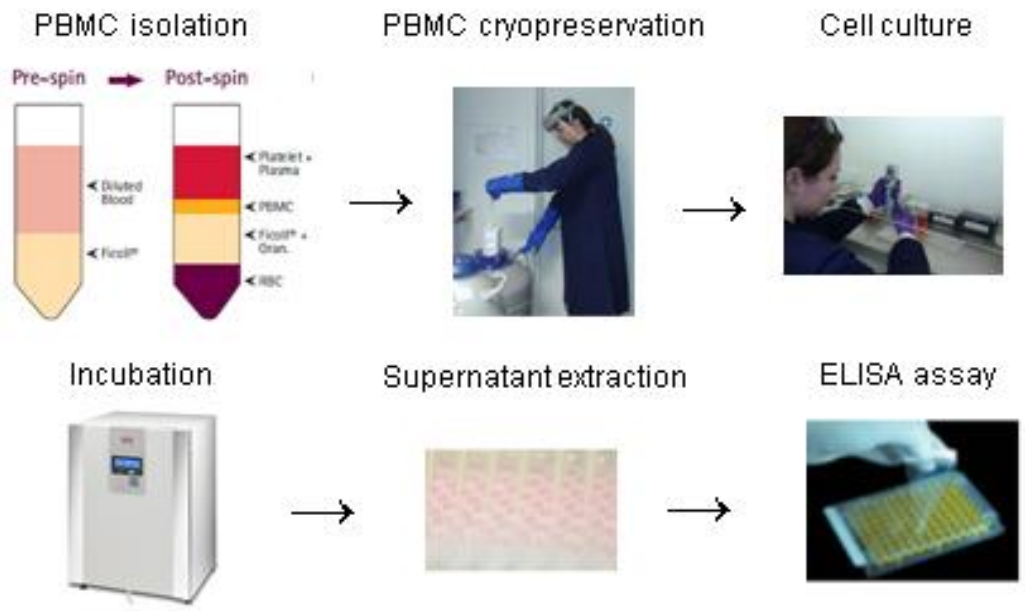


Figure 2.2 *In Vitro* GR Function Evaluation on PBMC (DEX-inhibition of LPS-stimulated IL-6 Production). Photographs of the author reproduced with kind permisson of Martha Villegas-Montes.

All the experiments in relation to GR function were performed at the University of Roehampton. Cell culture experiments were conducted in the microbiology laboratory containment level 2, and ELISA in the clinical laboratory, strictly observing the Health and Safety rules and regulations.

2.6 Tryptophan and Kynurenine Pathway Analysis

Tryptophan and kynurenine pathways were assessed by measuring the serum concentrations of tryptophan, kynurenine, and their metabolites. Serum samples were sent to Dr Aye Mu Myint and her colleagues in Germany (Laboratory for Psychoneuroimmunology, Ludwig-Maximilians University, Munich) to determine the levels of TRP, KYN, KYNA, 3-HK, and 3-HAA metabolites, using High performance liquid chromatography (HPLC) method. Based on their report, the measurements were performed according to the method of Herve et al. with some modifications (Herve et al., 1996). However, the recently published method using HPLC (Oades et al., 2010) was used to measure 3-HK. The following is a brief description of the method used: KYN was detected spectrophotometrically at 365 nm. KYNA was detected fluorimetrically at an excitation wavelength of 334 nm and an emission wavelength of 388 nm. KYNA was analyzed in plasma that was deproteinised using perchloric acid. 3-HK was measured at a wavelength of 365 nm by UV detection. All of the analyses were conducted using HPLC with a reversed phase C-18 column. The 3-HK analysis method has been validated showing an absolute recovery of 85.8%, intra-day precision of 3.9%, and inter-day precision of 7.5%. A time series demonstrated total stability of the analyte 3-HK during the extraction and analysis steps for up to 3x repeated freezing/thawing cycles, which ensured the validity of the analysis in samples that had already been thawed and frozen again. The intra- and inter-assay coefficients of variation ranged from 5% to 7% for all of the metabolites. The kynurenine to tryptophan (KYN/TRP) ratio was calculated by dividing the kynurenine (µg/ml) by the tryptophan levels (ng/ml).

2.7 Vascular Endothelial Growth Factor (VEGF) Measurement in Plasma

The quantitative determination of vascular endothelial growth factor (VEGF) concentrations in plasma samples was measured by commercially available ELISA kit (R&D systems, Minneapolis, MN, USA) (Rosti et al., 2007). The recommended procedure was followed. Briefly, the plasma samples were taken out of the freezer and thawed leaving them in room temperature. After adding 50 µl of Assay Diluent, 100 µl of standards, plasma samples, and controls (all assayed in duplicate) were pipetted into the appropriate wells of a microplate pre-coated by monoclonal antibody specific for VEGF. The plate was covered with the adhesive strip provided and was incubated for 2 hours in room temperature. The procedure allowed VEGF presented in samples to be bounded by the immobilized antibody. The plate were then washed using a plate washer to remove any unbound substances. After blotting the plate against clean paper towel to make sure removal of possible remaining wash buffer, 200 µl of VEGF conjugate (an enzyme-linked polyclonal antibody against VEGF conjugated to horseradish peroxidase) was added to each well, and the plate was incubated for 2 hours. After washing the plate to wash away any unbound antibody-enzyme reagent, 200 µl TMB solution was added to the wells and the plate was incubated for 25 minutes at room temperature and protected from light. The procedure allowed developing colour (from colourless to blue) in proportion to the amount of VEGF bound initially. The reaction was then stopped by adding 50 µl stop solution of sulfuric acid turning the colour from blue to yellow. The intensity of the colour was measured. Microplate reader (Beckman Coulter DTX 880) was used to determine optical density at 450 nm with correction at 570 nm. SoftMax Pro 4.8 software was used to calculate the VEGF values. Inter- and intra-assay variations were <10%.

2.8 Data Analysis

All statistical analyses were performed using SPSS software version 20.0 for Windows. The data were reported as the mean ± SEM. All data were tested for suitability for parametric or non-parametric analysis. In some cases such as CRP, the data with skewed distribution were log-transformed to correct the distribution for subsequent analysis. For comparing all the biological factors assessed in this study between the two groups of CHD patients without depression and CHD patients with depression, independent *t*-test was used (or Mann-Whitney U-test, as the non-parametric counterpart of the *t*-test, where specified). Dichotomous variables were compared using Chi-squared test for variables such as gender, marital and educational status, ethnicity, smoking statues, and also cardiac factors and concomitant medications. Correlations were assessed using Pearson's product moment correlation (or Spearman's correlation coefficient where the data violated parametric assumptions). For comparison of more than two groups, One-way ANOVA analysis (or its non-parametric counterpart Kruskal-Walis test) was carried out. A two-way ANOVA was used to determine how a response was affected by the two relevant factors. The analysis was performed on salivary cortisol repeated measurements as well as dexamethasone inhibition of LPS stimulated IL-6 levels. General linear model (GLM) was performed for taking into account the effect of covariates (ANCOVA) such as gender, age, and antidepressant usage on the findings. The p-values of <0.05 were considered as significant.

2.9 A Summary of Personal Contribution

I have optimized the cell culture protocol for measuring the sensitivity of GR. I recruited healthy controls, processed their blood, and isolated PBMC for the protocol optimization. I then conducted all the *in vitro* experiments in relation to GR sensitivity and the effects of drugs on the GR function in the patients. I also completed all ELISA assays to measure IL-6 levels in supernatant of the cultured cells.

For measuring cortisol and CRP levels, plasma and serum samples were sent to biochemistry laboratory, KingsPath, at King's College Hospital. I have then run all the data analysis in relation to these parameters.

I have contributed to the saliva samples collection by helping and supervising Jennie Parker who was a master student working in the INFLAME-BEAT study. Salivary cortisol measurements were conducted by Dr Patricia Zunszain at SPI-Lab, KCL. I, then, performed all the calculations of the CAR and AUC using formulas introduced by Pruessner and colleagues, followed by subsequent analyses.

Gene expression experiment was conducted by Dr. Izabela Barbosa at SPI-Lab, KCL. However, I performed all the subsequent calculations using Pfaffl method following by analyses to determine the Relative Expression Ratio of the candidate genes, and subsequent statistical analysis with the data.

The kynurenine and tryptophan pathway metabolites were measured by Dr Aye Mu Myint and her colleagues in Germany (Laboratory for Psychoneuroimmunology, Ludwig-Maximilians University, Munich). I have organized the transfer of samples from SPI-Lab

to Germany, and performed all required calculations and the subsequent statistical analyses after receiving the raw data.

I have run ELISA experiment for measuring VEGF levels followed by analyzing the data. And finally, I have organized the database of the biological data, ensuring data-quality against the master database and the hard-copy of the different findings.

Chapter 3 RESULTS

3.1 Characteristics of CHD Patients with and without Depression

The Characteristics of the CHD patients with depression (n=28) and without (n=55) are shown in the following tables.

Table 3.1 Socio-demographic characteristics of CHD patients with and without depression

	CHD (n=55)	**CHD-D (n=28)**	**Test and significance**
Age (years) (mean ± SEM)	70 ± 1	69 ± 2	t (81)=0.72, p=0.48
Gender (% of males)	89	54	χ^2 =13.26, **p=0.001****
Ethnicity (% of white)	91	82	χ^2 =1.35, p=0.25
Marital Status [n (%)]			
Married/civil partnership	41 (75%)	16 (59%)	χ^2 =2.28, p=0.52
Separated/divorced	5 (9%)	3 (11%)	
Widowed	4 (7%)	4 (15%)	
Single	5 (9%)	4 (15%)	
Educational Level [n (%)]			
≤10 years	23 (43%)	16 (59%)	χ^2 =2.00, p=0.16
>11 years	31 (57%)	11 (41%)	
Employment Status [n (%)]			
Employed	9 (16%)	3 (11%)	χ^2 =5.26, p=0.07
Retired/Housewife	44 (80%)	19 (70%)	
Unemployed	2 (4%)	5 (19%)	
Smoking Status [n (%)]			
Current	18 (33%)	9 (32%)	χ^2 =0.67, p=0.72
Ex	29 (53%)	13 (47%)	
Never	8 (14%)	6 (21%)	

Table 3.1 represents the socio-demographic characteristics of the patients. Except for gender, the two groups of CHD patients, with and without depression, did not differ in any demographic characteristics in terms of age, ethnicity, marital status, educational levels, employment, and smoking status. Due to the gender difference (χ^2 =13.26, **p<0.01), and specifically the fact that there were more female in depressed group than non-depressed (46% vs 11% respectively), the effect of gender has been analysed and taken into consideration as a covariant in all subsequent analysis.

Table 3.2 Psychometric characteristics of CHD patients with and without depression

	CHD (n=55)	CHD-D (n=28)
CIS-R (%)	0.0	100.0
BDI score (mean ± SEM)		
At the time of blood collection	4.2 ± 0.4	19.0 ± 1.3
At the time of saliva collection	4.3 ± 0.4	18.2 ± 1.7
History of Depression (%)	0	54
Family history of Depression (%)	13	36

CIS-R, Clinical Interview Schedule-Revised; BDI, Beck Depression Inventory

The Table 3.2 demonstrates the psychometric characteristics of the study population. The two main characteristics: CIS-R and BDI score, were used to define depression in CHD patients. As described previously, clinical diagnosis of depression was assessed based on the CIS-R. As shown in the table, all the CHD depressed patients (CHD-D) were clinically diagnosed with depression according to the CIS-R. In addition, all CHD-D subjects show depressive symptoms at the time of the sample collection that was quantified using the BDI (≥10). The information in regards to the history of depression and family history of depression were recorded by participant self-report at the baseline interview. 54% of CHD-D had a history of depression in the past, and 36% had a family history of depression. The control group, CHD without depression, were selected based on being CIS-R and BDI negative and also having no history of depression.

Table 3.3 Cardiac factors and diagnosis of CHD patients with and without depression

	CHD (n=55)	CHD-D (n=28)	Test and significance
Myocardial Infarction (%)	47	36	χ^2 =1.01, p=0.32
Hypertension (%)	69	79	χ^2 =0.92, p=0.34
Heart Rate (mean ± SEM)	58 ± 1	67 ± 4	t (52)=-2.09, **p=0.047***
Body Mass Index (BMI) (kg/m^2) (mean ± SEM)	29.17 ± 0.77	30.15 ± 1.54	U= 733.0, z= -0.23, p=0.82
High Cholesterol (%)	66	56	χ^2 =0.84, p=0.36
Family History of Heart Disease (%)	65	70	χ^2 =0.25, p=0.62
Diabetic CHD (%)	18	29	χ^2 =1.18, p=0.28

The Table 3.3 demonstrates cardiac related factors and diagnosis of the study participants. As presented, the two groups of CHD patients, with and without depression, had the same population with a history of myocardial infarction (p>0.05), the factor associated with increased risk of development of depression and cardiovascular mortality (Lichtman et al., 2008, Vasan et al., 2003). Some other cardiac factors, which have an unfavourable impact on inflammation and cardiovascular related events, have been also analysed with no difference being found between the two groups. These included hypertension defined as elevated systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg (p>0.05); BMI defined as weight (kg) divided by the square of height (m) (p>0.05); high cholesterol defined as elevated levels of total circulatory cholesterol ≥240 mg/dL (high density lipoprotein + low density lipoprotein + 20% triglyceride) (p>0.05); family history of heart problems defined as whether one or two of the patents and/or siblings developed heart problems (p>0.05) (Roger et al., 2011); as well as diabetes as diagnosed and recorded by their general practitioners (p>0.05).

As shown in the table, depressed group had significantly higher heart rate as compared to non-depressed CHD patients (*p<0.05). Although no significant difference was found between the two CHD groups regarding being on beta blocker therapy as presented in Table 3.4, there were more non-depressed (60%) than depressed (52%) taking beta blocker medications. Therefore, the findings in regards to heart rate were controlled for the effect of this medication using general linear model (F=1.807 p=0.185).

Table 3.4 Concomitant medications of CHD patients with and without depression

	CHD (n=55)	CHD-D (n=28)	Test and significance
Antidepressants (%)	0	39	
Statin (%)	89	80	χ^2 =0.99, p=0.32
ASA (%)	71	80	χ^2 =0.69, p=0.41
Anticoagulants (%)	21	20	χ^2 =0.01, p=0.91
Antihypertensive (%)	94	92	χ^2 =0.14, p=0.71
Beta-blockers (%)	60	52	χ^2 =0.40, p=0.53
ACE-inhibitors (%)	67	52	χ^2 =1.69, p=0.19
Ca^{2+}-channel inhibitors (%)	25	20	χ^2 =0.24, p=0.63

ACE, angiotensin-converting-enzyme inhibitor; ASA, acetylsalicylic acid

The concomitant medications of the study population are presented in the Table 3.4. As demonstrated in the table, CHD control group were free from antidepressant usage. However, 39.3% of CHD depressed group were taking antidepressants including SSRIs: fluoxetine, citalopram, sertraline, and TCAs: amitriptyline, nortriptyline, dosulepin. The effect of antidepressant have been analysed in biological system investigated in this PhD thesis by comparing depressed patients who are drug free and those who are on antidepressants. In both groups, almost all the patients were taking medications related to their cardiac condition, the most common of which are indicated in the table above. The cholesterol lowering statins included atorvastatin, simvastatin, rosuvastatin, and pravastatin. They were used in more than 80% of patients with no differences between the two CHD groups ($p>0.05$). The use of anti-inflammatory drug, acetylsalicylic acid (ASA), most commonly known as aspirin, also did not differ between CHD depressed and non-depressed ($p>0.05$). No differences were found in taking warfarin and clopidogrel which were two anticoagulant medications in the two study groups ($p>0.05$). More than 90% of the subjects in each group were taking antihypertensive medications

including β-blockers (atenolol, bisoprolol, carvedilol, metoprolol, propranolol, nebivolol), angiotensin-converting-enzyme (ACE)-inhibitors (ramipril, lisinopril, enalapril, perindopril), and Ca^{2+}-channel inhibitors (amlodipine, nifedipine, felodipine, diltiazem). There were no significant differences between groups in taking antihypertensive medications in total as well as in each subtype of drugs ($p>0.05$) as indicated in the table.

3.2 Inflammation in CHD Patients with and without Depression

3.2.1 Inflammatory Biomarker: C - Reactive Protein (CRP)

In order to investigate the degree of inflammation in the study population, the present study firstly focused on the CRP levels in both CHD patients with and without depression, and compared the circulatory levels of CRP in serum between the two groups.

CRP is the most sensitive acute phase reactant and a marker of on-going inflammation (Willerson and Ridker, 2004). CRP as a clinical biomarker of inflammation has been found to be a robust predictor of future cardiac events, even stronger than LDL cholesterol (Ridker, 2003). In addition, elevation of CRP which is implicated in the pathogenesis of CHD (Huffman et al., 2013), has been also shown to be associated with presence of the depressive symptoms in otherwise healthy individuals (Miller et al., 2002).

According to the American Heart Association, the CRP levels <1, 1-3, and, >3 mg/L are, respectively, low, intermediate, and high risk factors for cardiovascular events. Measuring the circulating serum levels of CRP in the present study population, as shown in Figure 3.1, demonstrated that both CHD groups exhibited CRP levels more than 3 mg/L, and so being in high risk group (CHD 3.34 ± 0.75 mg/L and CHD-D 5.21 ± 0.99 mg/L). However, inflammation was found to be even higher (>5 mg/L) in CHD patients with depression.

The CRP values were tested for normality, and since the data were not normally distributed, they were log transformed (log10) to improve suitability for parametric

statistics. CRP concentrations were significantly higher in CHD-D when compared with CHD control group: Log CRP (mean ± SEM); CHD 0.096 ± 0.12, CHD-D 0.509 ± 0.12, t (52) = -2.361, **p=0.016**. However, for the presentation purposes, the raw values were used to illustrate the actual levels of CRP as shown in Figure 3.1.

CRP was found to be positively correlated with the BDI scores (r_s=.342, **p=.012**, n=53) indicating the association of inflammation with the severity of depressive symptoms in CHD patients with and without depression (Figure 3.2). In addition, CRP was found to be associated with higher heart rate in these patients (r_s=.373, **p=.008**, n=50).

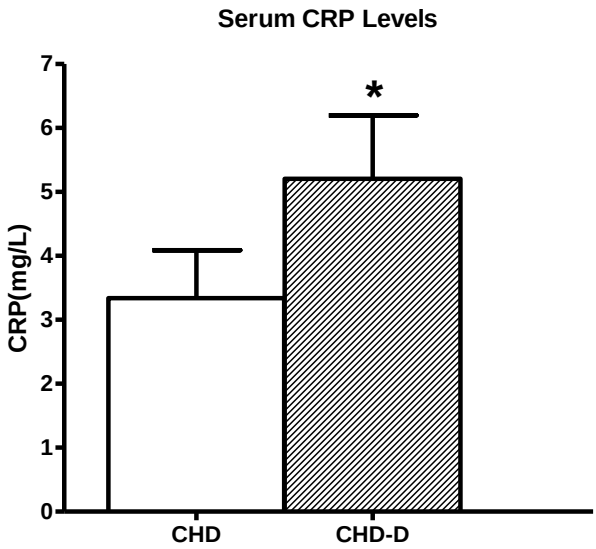


Figure 3.1 Circulating CRP concentrations (mg/L) in CHD patients with and without depression. Data expressed as mean ± SEM, (CHD, n=33; CHD-D, n=21).

CHD patients with and without depression had high levels of CRP (>3 mg/L) and both are at high risk group for cardiovascular events. Compared to non-depressed group (CHD, n=33), CHD patients with depression (CHD-D, n=21) showed a statistically significant elevation in serum CRP indicating even higher levels of inflammation (*$p<0.05$).

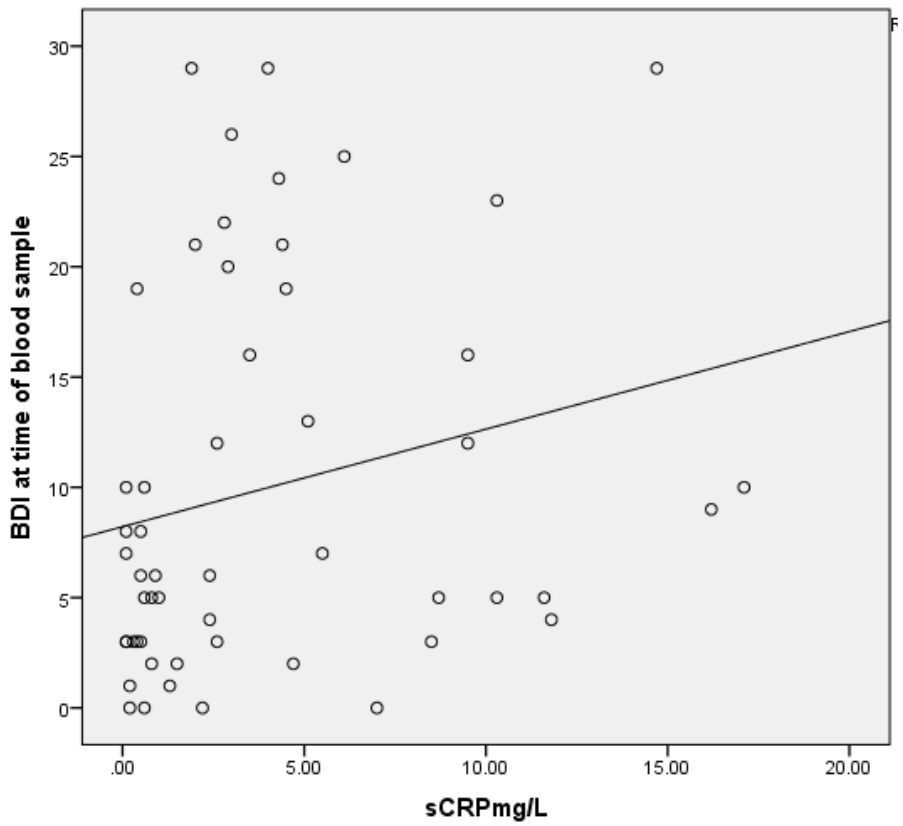


Figure 3.2 Association of CRP concentrations with BDI scores in CHD patients with and without depression (r_s=.342, *p<.05, n=53).

Serum CRP levels (mg/L) were significantly associated with the BDI scores in CHD patients with and without depression (r_s=.342, *p<.05, n=53). The positive correlation indicates that higher inflammation was associated with more severe depressive symptoms in these patients.

3.2.2 Gene Expression and Inflammation

Gene expression profile is suggested to be a useful tool developed for identifying molecular biomarkers associated with diseases (Sunde, 2010). Whole blood is considered as an ideal tissue for measuring the expression levels of genes due to its feasible obtainability. This is especially relevant in advancing neuropsychiatric research where the CNS tissue is rather inaccessible (Sullivan et al., 2006). Since there is a constant interaction between blood cells and the entire body, alteration in gene expression at the peripheral level may reflect the changes of the particular tissue associated with the disorder (Liew et al., 2006). Studies show expression of a large proportion of the genes, approximately 80%, by peripheral blood cells which in turn share over 80% similar gene expression profiles when compared with a range of different major tissues including the brain (Liew et al., 2006, Sullivan et al., 2006, Sunde, 2010). Indeed, peripheral blood cells are being used to peruse transcriptomic research in major depression (Mehta et al., 2010). Pro-inflammatory changes in relation to depression have been also studied through genome expression analysis at the peripheral level (Savitz et al., 2013).

For the purpose of this PhD thesis, in addition to measuring circulatory levels of CRP in the study population, inflammation has been also investigated through gene expression analysis of candidate genes involved in inflammatory response that included: IL-6, IL-1β, NF-kβ, and TNF-α.

As described before in section 1.2.8 antidepressants exert beneficial effect in modulation of inflammation and specifically studies suggest their role in inhibition of production and release of pro-inflammatory cytokines including IL-1, IL-6, and TNF-α (Tousoulis et al., 2009, Xia et al., 1996). Therefore, considering the fact that almost 40% of CHD-D patients were on antidepressant treatment, it was interesting to investigate the *in vivo*

effects of antidepressants on the expression of the candidate genes by comparing depressed patients who were drug free (CHD-D-noAD) with those on antidepressants (CHD-D-AD).

3.2.2.1 Gene Expression of Interleukin-6 (IL-6)

In order to evaluate further the inflammatory response, gene expression levels of IL-6 were analysed. IL-6 encoded by IL-6 gene is a circulating pro-inflammatory cytokine and a potent inducer of acute phase responses that regulates hepatic synthesis and release of CRP (Heinrich et al., 1990, Yudkin et al., 2000). Although hepatic acute phase reactions are regulated also by other pro-inflammatory cytokines, IL-6 plays a primary role in this regard. Indeed, as evidenced by animal studies, compared to IL-1 and TNF-α deficient models, IL-6 knockout animals are the ones exhibiting acute phase response impairment (Maes et al., 2011, Sherwood et al., 2007).

IL-6 is known as a powerful predictor of chronic and low-grade inflammatory state (Rohleder et al., 2012). This pro-inflammatory cytokine and its signalling events contribute to prognosis of atherosclerosis (Schuett et al., 2009). In addition, IL-6 in the context of inflammation is a major mediator of CNS-immune interaction and its elevation has been shown to be closely linked in chronic stress as well as fatigue and sleep disturbances (Rohleder et al., 2012). Furthermore, increased IL-6 and its direct effect on the brain are implicated in the pathophysiology of depression (Glassman et al., 1993).

Therefore, in the present thesis, IL-6 gene expression was considered as an important candidate gene to be investigated in the study population. As illustrated in Figure 3.3, IL-6 mRNA expression was shown to be significantly increased in CHD depressed patients compared to non-depressed ($t=3.793$, $df=37$, **$p=0.0005$**).

The effects of antidepressant treatment on IL-6 gene expression are presented in Figure 3.4. Both groups of CHD depressed patients either on antidepressant treatment or drug-free, showed significantly higher levels of IL-6 gene expression as compared to CHD patients without depression (One-way ANOVA, $F(2,36)=8.083$, **p=0.0013**). Antidepressant use had no significant effect on the gene expression of IL-6 in CHD patients with depression compared to antidepressant-free CHD depressed individuals ($t=0.8170$, $df=14$, **p=0.428**).

Gender and age were found to have a significant effect on IL-6 expression; $F(1,36)=4.226$, **p=0.047** and $F(1,36)=6.167$, **p=0.018**, respectively. However, the result held true after controlling for the effect of these covariates ($F(3,35)=4.538$, **p=0.04**).

As presented in Figure 3.5, the result on the association between the pro-inflammatory cytokine IL-6 and severity of depression showed a positive correlation between IL-6 gene expression and depressive symptomatology quantified by BDI scores ($r =.356$, **p=.026**, $n=39$).

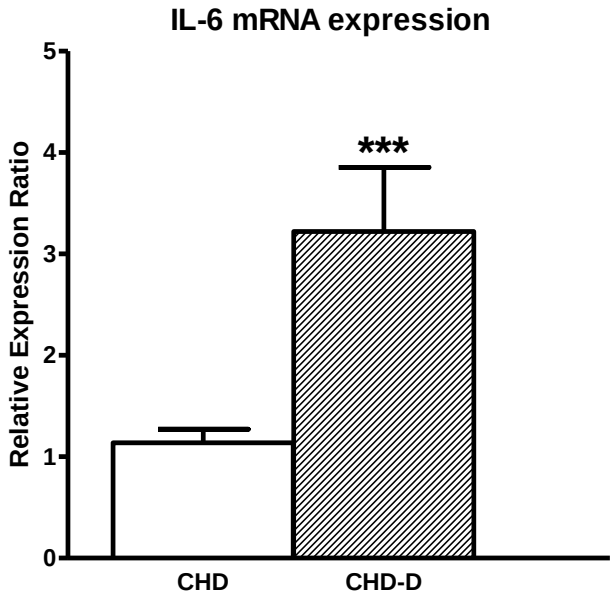


Figure 3.3 IL-6 mRNA expression in CHD patients with and without depression. Data expressed as mean ± SEM, (CHD, n=23; CHD-D, n=16).

CHD patients with depression (CHD-D, n= 16) exhibited significantly increased in IL-6 expression as compared to CHD patients without depression (CHD, n=23) (***$p<0.001$).

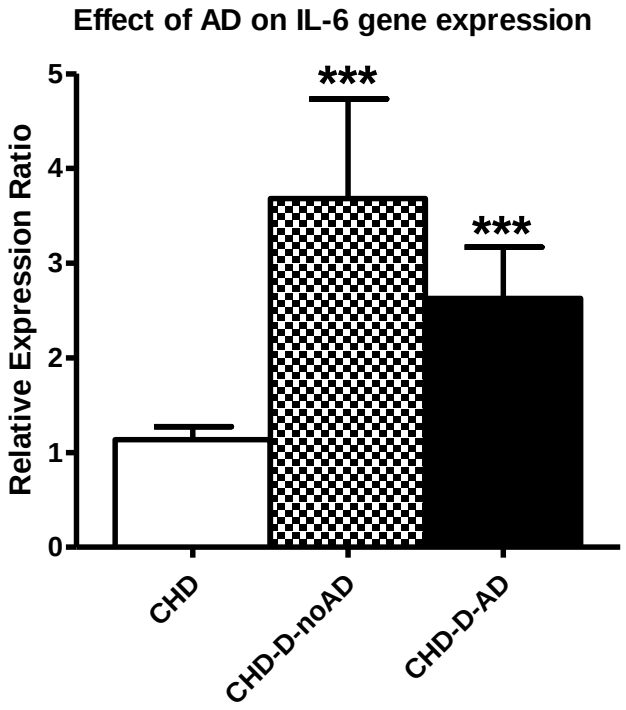


Figure 3.4 The effect of antidepressants on IL-6 gene expression. Data expressed as mean ± SEM, (CHD, n=23; CHD-D-noAD, n=9; CHD-D-AD, n=7).

CHD depressed patients with (CHD-D-AD, n=7) and without (CHD-D-noAD, n=9) antidepressant treatment showed significantly higher levels of IL-6 gene expression as compared to CHD patients without depression (CHD, n=23) (***$p<0.001$). Antidepressant use had no significant effect on the gene expression of IL-6 in CHD patients with depression compared to antidepressant-free CHD depressed individuals (CHD-D-AD vs CHD-D-noAD, $p>0.05$).

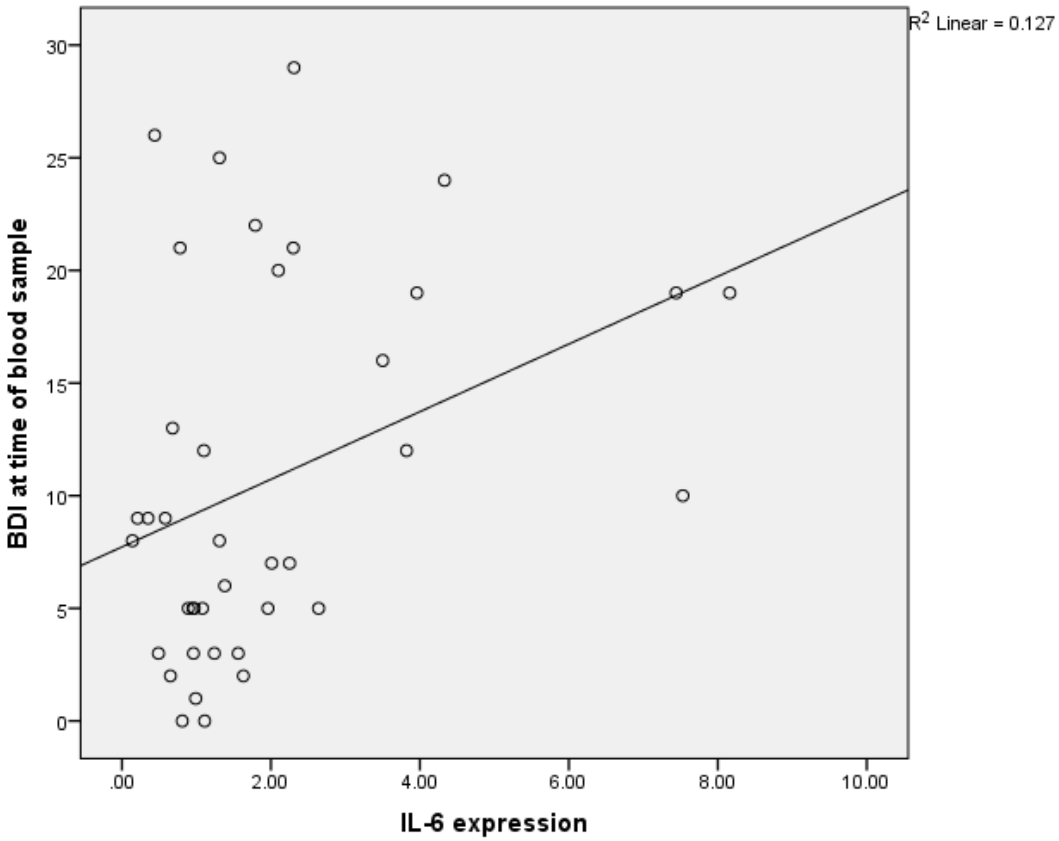


Figure 3.5 Association of IL-6 expression with BDI scores in CHD patients with and without depression (r =.356, *p<.05, n=39).

IL-6 gene expression was significantly associated with the BDI scores in CHD patients with and without depression (r =.356, *p<.05, n=39). The positive correlation indicates that increase in expression of inflammatory cytokine IL-6 were associated with more severe depressive symptoms in these patients.

3.2.2.2 Gene Expression of Interleukin-1beta (IL-1β)

IL-1β was another inflammatory cytokine whose expression was assessed in this PhD. IL-1β encoded by IL-1β gene is a member of IL-1 family and classified as type 1 cytokines involved in enhancing cellular immune responses. This potent inducer of innate immune response stimulates the expression and release of secondary inflammatory mediators. IL-1β is the main circulating IL-1 involved in systemic inflammatory response.

An increased level of this cytokine has been reported in inflammatory disorders and CHD (Hansson, 2005a, Stegger et al., 2012). Indeed, IL-1β has been suggested to be involved in development of atherosclerotic arteries (Olofsson et al., 2009) and incidence of myocardial infarction (Iacoviello et al., 2005). On the other hand, elevation of IL-1β is observed in MDD, late-life depression, and treatment resistant depression (Maes et al., 1993, Maes et al., 1997, Thomas et al., 2005).

Therefore, IL-1β gene expression was assessed as another candidate gene. As illustrated in Figure 3.6, there was no difference in IL-1β mRNA expression in CHD patients with and without depression (t=0.277, df=38, **p=0.784**).

The effect of antidepressants treatment on IL-1β gene expression is presented in Figure 3.7. The graph shows that antidepressants appeared to decrease the gene expression of IL-1β in CHD patients with depression compared to antidepressant-free CHD depressed individuals, but the effect was not found significant (One-way ANOVA: F(2,37)=2.147, **p=0.132**; CHD-D-noAD vs CHD-D-AD t=1.667, df=14, **p= 0.118**).

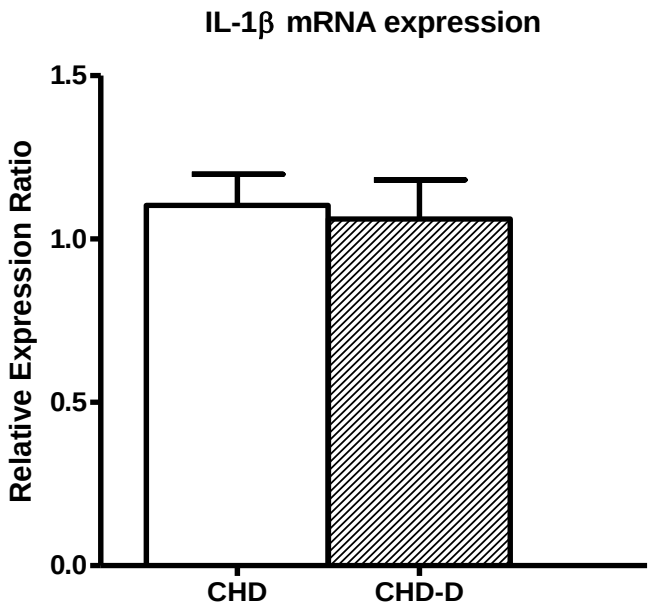


Figure 3.6 IL-1β mRNA expression in CHD patients with and without depression. Data expressed as mean ± SEM, (CHD, n=24; CHD-D, n=16).

There was no difference in the levels of IL-1β gene expression when CHD patients with depression (CHD-D, n=16) were compared with CHD patients without depression (CHD, n=24) ($p>0.05$).

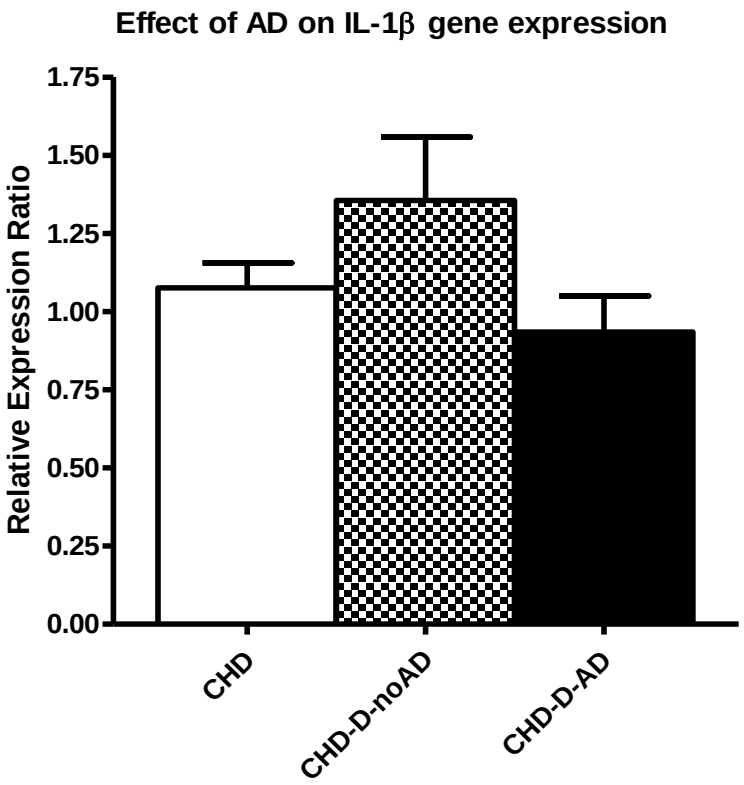


Figure 3.7 The effect of antidepressants on IL-1β gene expression. Data expressed as mean ± SEM, (CHD, n=24; CHD-D-noAD, n=9; CHD-D-AD, n=7).

Antidepressants appeared to decrease the gene expression of IL-1β in CHD patients with depression (CHD-D-AD, n=7) compared to antidepressant-free CHD depressed individuals (CHD-D-noAD, n=9), but the effect was not significant ($p>0.05$).

3.2.2.3 Gene Expression of Tumour Necrosis Factor-alpha (TNF-α)

Gene expression of TNF-α was assessed in this PhD thesis, because this cytokine also is known to be involved in systemic inflammation and triggering acute phase reactants (Popa et al., 2007). As the name suggests, the cytokine was first recognized as an endotoxin induced serum factor triggering the necrosis of tumours (Carswell et al., 1975). TNF-α is encoded by TNF gene, and its expression, synthesis, and release into the circulation are induced in response to inflammatory stimuli (Kriegler et al., 1988). This pro-inflammatory cytokine plays a role in regulating the expression of IL-6 and its elevation has been observed in chronic inflammatory conditions (Bradley, 2008, Suarez-Cuervo et al., 2003).

The studies on TNF-α in relation to heart disease suggest the implication of increased circulatory levels of the cytokine in pathophysiology of cardiac related conditions specifically in the development of atherosclerosis and the progression of chronic heart failure (Feldman et al., 2000, Sack, 2002). In relation to depression, a recent meta-analysis of 24 published studies reported significantly higher concentration of this pro-inflammatory cytokine along with IL-6 in MDD patients as compared to healthy controls (Dowlati et al., 2010).

Therefore, TNF-α gene expression was assessed as another candidate gene. As illustrated in Figure 3.8, there was no difference in TNF-α mRNA expression in CHD patients with and without depression (t=0.449, df=38, **p=0.656**). The effect of antidepressants treatment on TNF-α gene expression is presented in Figure 3.9. Antidepressants had no significant effect on gene expression of TNF-α in CHD patients with depression compared to antidepressant-free CHD depressed individuals (One-way ANOVA: F(2,37)=0.191, **p=0.827**); CHD-D-noAD vs CHD-D-AD t=0.506, df=14, **p= 0.621**).

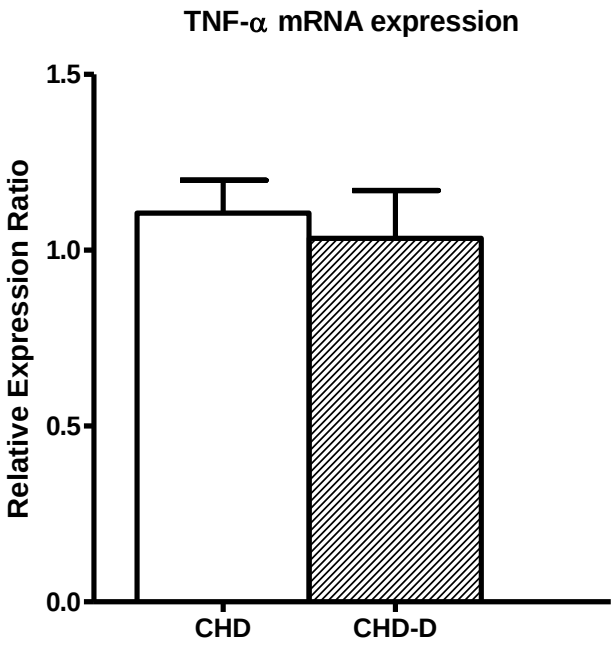


Figure 3.8 TNF-α mRNA expression in CHD patients with and without depression. Data expressed as mean ± SEM, (CHD, n=24; CHD-D, n=16).

CHD patients with depression (CHD-D, n=16) showed no difference in the levels of TMF-α gene expression when compared with CHD patients without depression (CHD, n=24) ($p>0.05$).

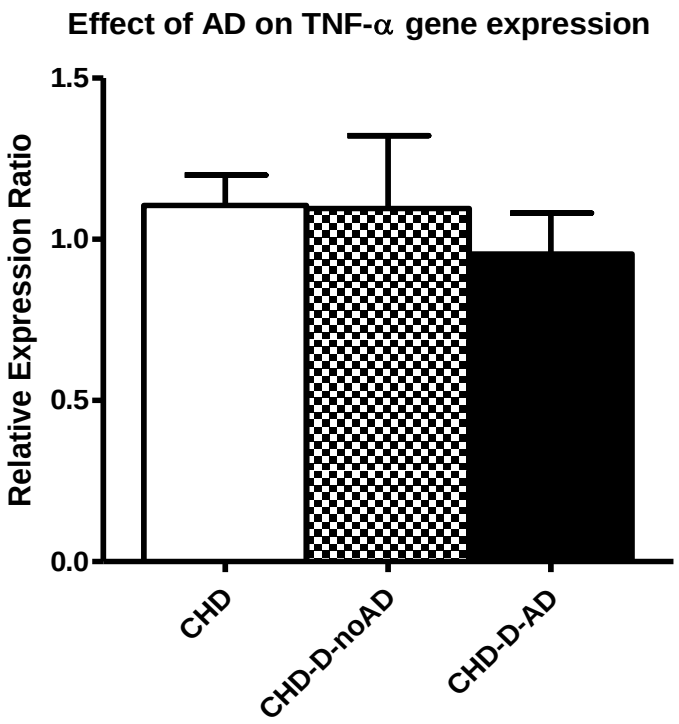


Figure 3.9 The effect of antidepressants on TNF-α gene expression. Data expressed as mean ± SEM, (CHD, n=24; CHD-D-noAD, n=9; CHD-D-AD, n=7).

Antidepressants treatment had no significant effect on gene expression of TNF-α in CHD patients with depression (CHD-D-AD, n=7) when compared with antidepressant-free CHD depressed individuals (CHD-D-noAD, n=9) ($p>0.05$).

3.2.2.4 Gene Expression of Nuclear Factor-kappaB (NF-kB)

NF-kB is one of the most important transcription factors involved in regulation of immune and inflammatory responses. While activated in response to inflammatory cytokines IL-1 and TNF-α, NF-kB is one of the main regulators and mediators of gene expression and synthesis of pro-inflammatory cytokine including IL-1, IL-6, and TNF-α (Baldwin, 1996). Inappropriate activation of NF-kB plays a key role in pathogenesis of inflammatory diseases (Tak and Firestein, 2001).

NF-kB has been identified to be implicated in cardiac related conditions including its involvement in initiation and progression of atherosclerotic plaque, development of unstable angina, and heart failure (Valen et al., 2001). In regards to depression, animal studies reveal the association between activation of NF-kB (and also increased IL-1β, TNF-α, IL-6) and onset of stress-induced depressive like behaviour (Kubera et al., 2011).

Therefore, NF-kB gene expression was assessed as another candidate gene. As illustrated in Figure 3.10, there was no difference in NF-kB mRNA expression in CHD patients with and without depression (t=0.591, df=38, **p=0.559**). The effect of antidepressants treatment on NF-kB gene expression is presented in Figure 3.11. As shown, antidepressants had no significant effect on gene expression of NF-kB in CHD patients with depression compared to antidepressant-free CHD depressed individuals (One-way ANOVA, F(2,37)=0.432, **p= 0.653**); CHD-D-noAD vs CHD-D-AD t=0.652, df=14, **p= 0.525**).

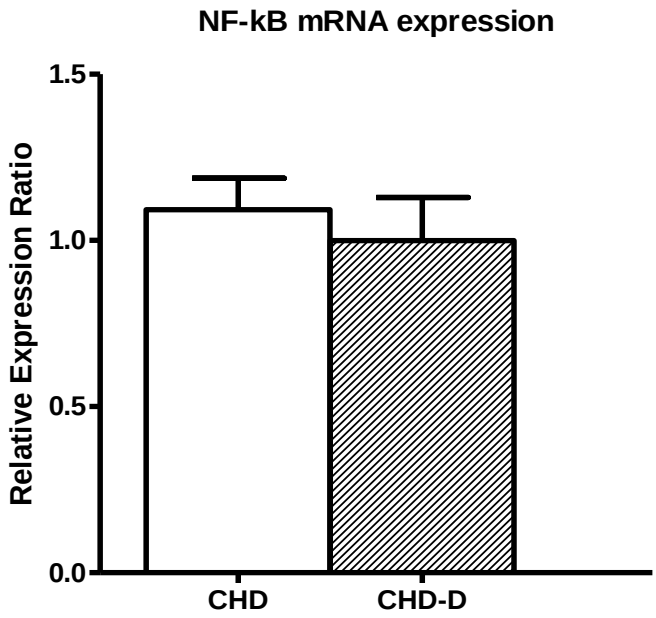


Figure 3.10 NF-kB mRNA expression in CHD patients with and without depression. Data expressed as mean ± SEM, (CHD, n=24; CHD-D, n=16).

CHD patients with depression (CHD-D, n=16) showed no difference in the levels of NF-kB gene expression when compared with CHD patients without depression (CHD, n=24) ($p>0.05$).

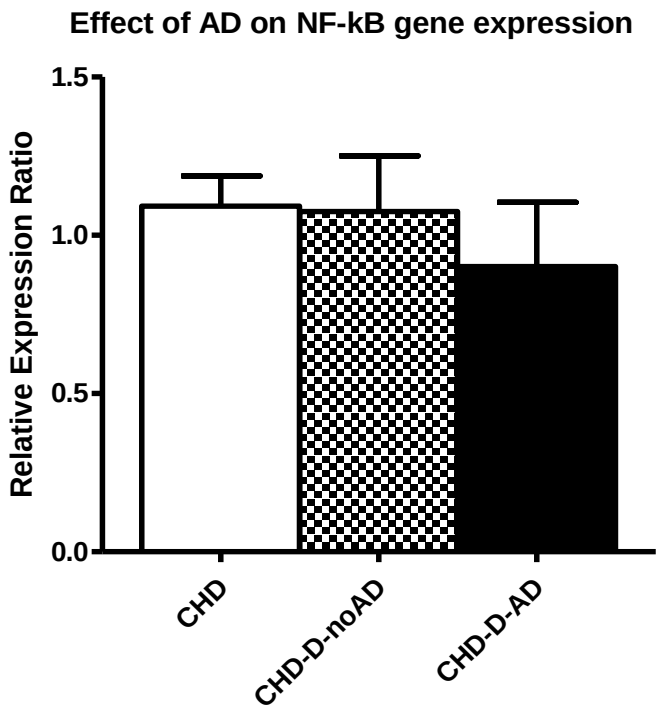


Figure 3.11 The effect of antidepressants on NF-kB gene expression. Data expressed as mean ± SEM, (CHD, n=24; CHD-D-noAD, n=9; CHD-D-AD, n=7).

Antidepressants treatment had no significant effect on gene expression of NF-kB in CHD patients with depression (CHD-D-AD, n=7) when compared with antidepressant-free CHD depressed individuals (CHD-D-noAD, n=9) ($p>0.05$).

3.3 Cortisol in CHD Patients with and without Depression

Having investigated the inflammatory response and the findings in regards to high levels of circulatory CRP and increased IL-6 gene expression in this PhD project, the next step was assessing the HPA axis as the main physiological circuit involved in regulation of stress induced inflammatory responses, through measuring the levels of endogenous glucocorticoid cortisol as an end product of the system. HPA axis, its function and regulation were described in details in section 1.2.2.

There is extensive evidence indicating the involvement of the HPA system in the pathophysiology of stress-related disorders including MDD that was discussed in section 1.2.4. In the other hand, HPA axis dysregulation and its implication in cardiovascular diseases has been also reported by various studies (Kumari et al., 2011, Reynolds et al., 2010, Yamaji et al., 2009).

Therefore, in order to elucidate possible disturbances in the HPA axis activity in the present population the circulatory concentrations of cortisol in plasma as well as free cortisol levels in saliva were measured.

3.3.1 Plasma Cortisol

Circulatory cortisol levels in plasma were measured and compared in CHD patients with and without depression. As demonstrated in Figure 3.12, CHD patients with depression had lower plasma cortisol than non-depressed group; cortisol (mean ± SEM); CHD 354.7 ± 19.28 nmol/L, CHD-D 280.6 ± 26.66 nmol/L, t (55) = 2.285, **p=0.026**. The effect of antidepressants treatment on plasma cortisol has been also investigated and is presented in Figure 3.13. As shown, antidepressants had no significant effect on plasma cortisol concentration in CHD patients with depression compared to antidepressant-free CHD depressed individuals (One-way ANOVA, F (2,54)= 2.652, **p= 0.080**); CHD-D-noAD vs CHD-D-AD t=0.3835 df=19, p= 0.706).

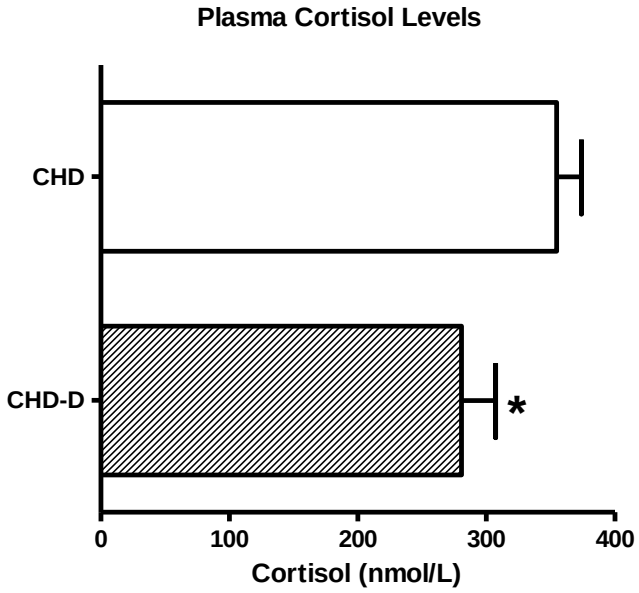


Figure 3.12 Plasma Cortisol levels (nmol/L) in CHD patients with and without depression. Data expressed as mean ± SEM, (CHD, n=36; CHD-D, n=21).

Compared to CHD patients without depression (CHD, n=36), depressed CHD individuals (CHD-D, n=21) showed significantly lower circulatory levels of cortisol (nmol/L) as measured in plasma (*$p<0.05$).

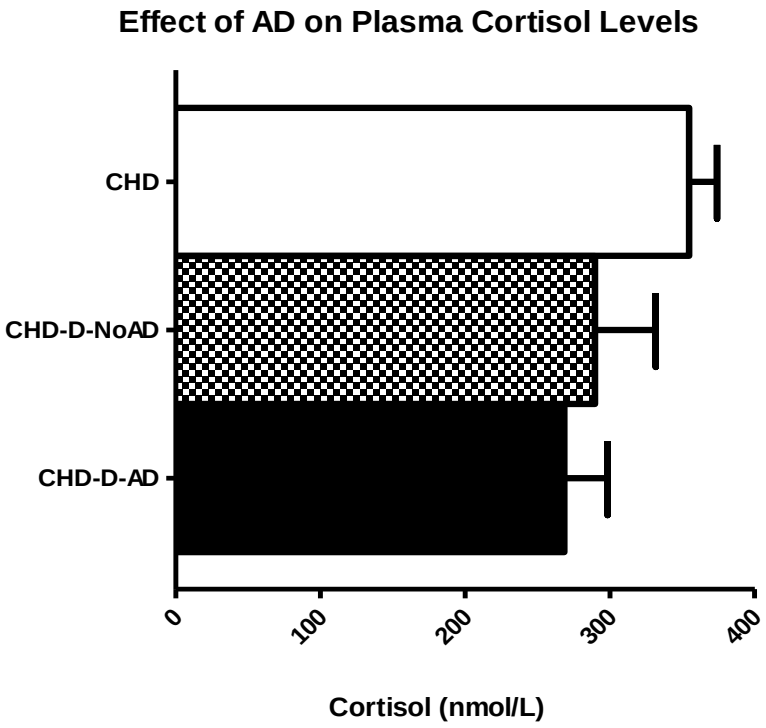


Figure 3.13 The effect of AD on the plasma cortisol levels. Data expressed as mean ± SEM, (CHD, n=36; CHD-D-noAD, n=12; CHD-D-AD, n=9).

Antidepressants treatment had no effect on the levels of plasma cortisol (nmol/L) in CHD patients with depression (CHD-D-AD, n=9) when compared with antidepressant-free CHD depressed individuals (CHD-D-noAD, n=12) ($p>0.05$).

3.3.2 Salivary Cortisol

In a healthy response, secretion of cortisol follows pronounced circadian rhythms which is characterized by marked increased in the morning shortly after awakening over the first hour with the peak at around 30 minutes (up to 50 to 60%), and then steadily decrease over the day within which short-term increases can be observed in response to normal daily stimuli such as meal, exercise, and other stressors (Tsigos and Chrousos, 2002, Wust et al., 2000). The very distinctive post awakening pattern of cortisol is known as cortisol awakening response (CAR) which is studied also together with the pattern of cortisol secretion over the day as indicators of adrenocortical functioning. Modification of diurnal cortisol profile is thought to be associated with pathophysiological changes in stress related disorders and immune response activity, and serves as an index to identify HPA axis disturbances (Pruessner et al., 1997, Wilhelm et al., 2007).

Cortisol circadian rhythm can be examined by measuring the free adrenal hormone in both blood and saliva samples showing the same diurnal pattern (Aardal and Holm, 1995). However, salivary cortisol sampling has been the preferred method over blood sampling in clinical studies and research settings as being an easy to employ, inexpensive, non-invasive, laboratory independence and stress-free way to collect samples especially from home-based patients and widely dispersed populations (Clow et al., 2004, Kirschbaum and Hellhammer, 1999). Therefore, in the present thesis, apart from a single assessment of cortisol in blood sample to determine the circulatory plasma concentration and compare the levels in the two groups of CHD patients with and without depression, salivary cortisol samples were also obtained over the day to assess the HPA axis activity through evaluating diurnal cortisol circadian rhythm.

Obtaining repeated measurements of salivary cortisol and then calculating the area under the curve of the measures or AUC (section 2.3) provided two types of information embodied in the multivariate data by transforming them into univariate form. AUC_G, being calculated with reference to the ground, revealed information in regards to the total cortisol output by measuring the total area under the curve considering the distance of the measures from the ground and therefore providing information regarding intensity and magnitude of the cortisol response. Whereas, AUC_I, being calculated with reference to the increase from the baseline measurement revealed information in regards to sensitivity of the response by measuring the cortisol changes over time taking into account the distance of each measure from its neighbour (Pruessner et al., 2003).

The response of cortisol to the awakening is presented in Figure 3.14. Results showed that CHD patients with depression exhibited significantly decreased cortisol levels in the first hour after awakening compared to CHD non-depressed (Two-way ANOVA, $F(1,3)= 19.45$, $n=51$, **$p<0.0001$**). The lower cortisol concentrations were observed for each measure taken at 0, 15, 30, 60 min post awakening specially with the significant findings at 30 min ($t=2.732$, **$p<0.05$**) and 60 min ($t=3.149$, **$p<0.01$**) obtained by performing Bonferroni post-tests. The results from calculating the area under the curve for the cortisol awakening response in respect to the ground (distance from zero) also revealed significantly lower cortisol output during the first hour post awakening: CAR AUC_G, (mean ± SEM); CHD 724.3 ± 60.80, CHD-D 452.1 ± 65.54, $t (51) = 2.663$, **$p=0.010$**).

In addition, CHD depressed showed a blunted HPA axis reactivity as indicated by the blunted cortisol response to the awakening compared to CHD without depression. The response was calculated first as a difference between the cortisol levels at 30 minutes after awakening when the peak is expected, and those at the awakening (delta cortisol

levels at 30 minutes). The depressed patients had lower delta cortisol levels at 30 minutes post awakening compared with non-depressed group: Δ30min (mean ± SEM); CHD 5.26 ± 0.63, CHD-D 3.15 ± 0.68, t (51) = 2.029, **p=0.048**). Indeed, calculating the area under the curve taking into account the measurements of the first hour post awakening in respect to the increase from the baseline measurement (awakening) showed decreased cortisol response and so diminished responsiveness of HPA axis to the stress of awakening in CHD depressed compared to non-depressed group: CAR AUC_I (mean ± SEM); CHD 228.3 ± 27.67, CHD-D 132.9 ± 30.58, U (51) = 2.076, **p=0.043**).

Having found high levels of inflammation as indicated by CRP levels (section 3.2.1), and blunted HPA axis activity as indicated by blunted cortisol response, CRP was found to be negatively associated with CAR AUC_I (r=-0.429, **p=0.029**). The result is illustrated in Figure 3.15 showing that lower HPA axis responsiveness is associated with higher inflammation.

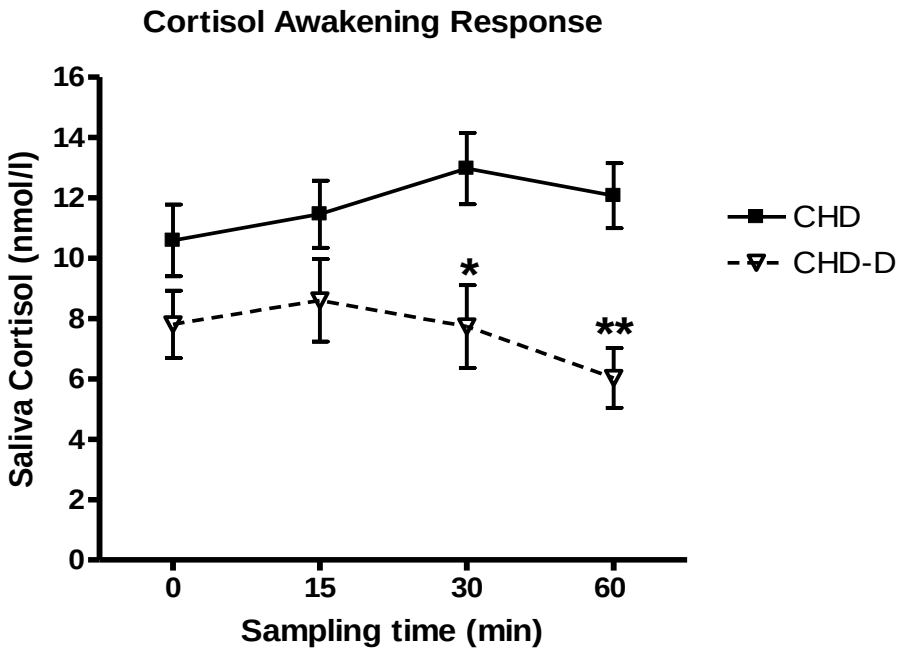


Figure 3.14 Mean salivary cortisol levels for the first hour after awaking at four sampling points on awakening and 15, 30, and 60 mins later in CHD patients with and without depression. Data expressed as mean ± SEM, (CHD, n=37; CHD-D, n=16).

Compared to CHD patients without depression (CHD, n=37) as indicated by solid line, depressed CHD individuals (CHD-D, n=16) as indicated by dotted line, showed significantly lower levels of free cortisol (nmol/l) in the first hour after awakening as measured in four time points (0, 15, 30, and 60 min) in saliva ($p<0.0001$). Overall cortisol output in the first hour post awakening was decreased in CHD depressed compared to non-depressed group calculated by area under the curve: CAR AUC_G ($p<0.05$). In addition, CHD depressed patients exhibited blunted cortisol response when compared with CHD non-depressed: CAR AUC_I ($p<0.05$). (*$p<0.05$) (**$p<0.01$)

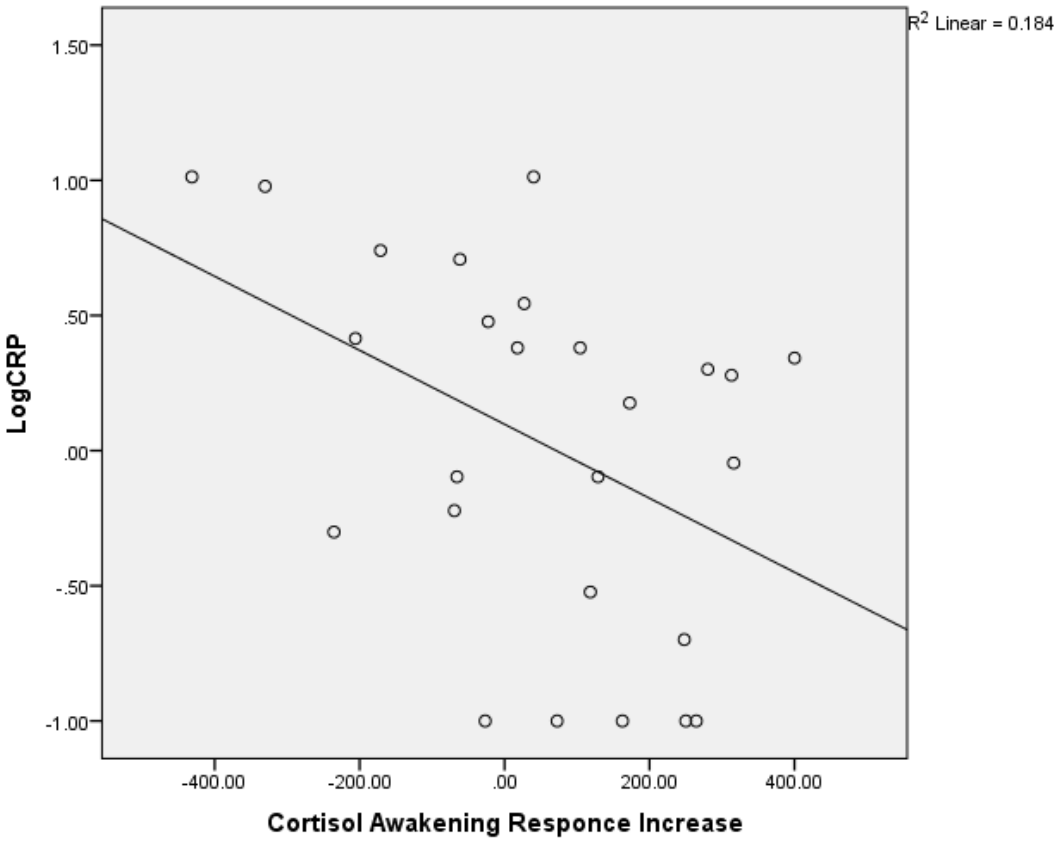


Figure 3.15 Negative association of CAR with CRP in CHD patients with and without depression (r=-0.429, *p<.05, n=26).

Cortisol awakening response (CAR_I) was negatively associated with CRP (r=-0.429, *p<.05, n=26). The negative correlation indicates that lower in HPA responsiveness were associated with higher inflammation in CHD patients with and without depression.

Diurnal cortisol was also assessed (Figure 3.16). Although as illustrated in the figure, cortisol levels showed a decline at each time point (awakening, 2 pm, and bedtime) of sampling in CHD patients with depression compared to CHD without depression, the result did not reach the statistical significance, and it only showed a trend of decreased cortisol concentration in CHD depressed individuals (Two-way ANOVA, F=3.390, **p=0.068**). The area under the curve for the diurnal cortisol was also calculated with the result showing no significant difference in the overall cortisol output during the day when calculated with the reference to the ground: Diurnal AUC_G: U (51) = 225.0, **p= 0.172**. Calculating the area under the curve with respect to the increase also revealed no significant difference when comparing CHD patient with and without depression: Diurnal AUC_I: U (51) = 275.0, **p= 0.691**. No difference in awakening time was found between the two groups: (mean ± SEM); CHD 7:04 ± 0:09, CHD-D 7:03 ± 0:22, t (51) = 0.013, **p=0.990**).

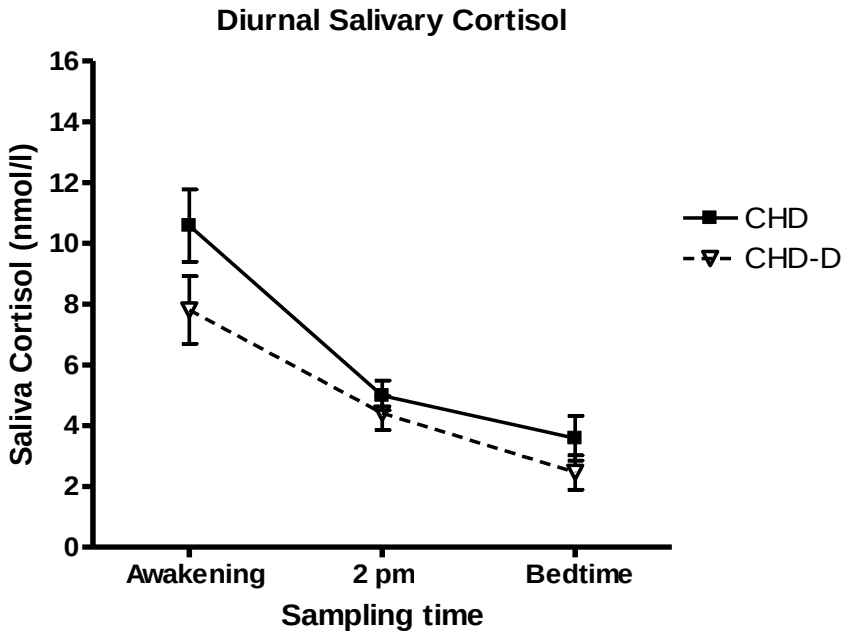


Figure 3.16 Mean salivary cortisol levels over the day presented at three sampling points on awakening, 2 pm, and Bedtime in CHD patients with and without depression. Data expressed as mean ± SEM, (CHD, n=37; CHD-D, n=16).

Compared to CHD patients without depression (CHD, n=37) as indicated by solid line, depressed CHD individuals (CHD-D, n=16) as indicated by dotted line, showed a trend but not significantly decrease the levels of diurnal cortisol (nmol/l) as measured in three time points during the day (awakening, 2 pm, and bedtime) in saliva ($p=0.068$). Overall diurnal cortisol did not differ in CHD patients with and without depression as calculated by area under the curve: Diurnal cortisol AUC_G ($p>0.05$).

3.4 Glucocorticoid Receptor (GR) in CHD Patients with and without Depression

GR and its involvement in inflammation and depression were discussed in details in sections 1.2.3 and 1.2.4. As mentioned before, the crucial immunosuppressive and anti-inflammatory role of GC is in fact through GR mediated transrepression activity targeting the genes associated with inflammatory cytokines (De Bosscher and Haegeman, 2009). Indeed, GR sensitivity to GC is crucial in order to produce an appropriate response that is determined by the number, affinity, and function of the receptor. Therefore, any alteration in GR number, its binding affinity, and/or functional capacity results in alteration of GR mediated GC response (Marques et al., 2009). Indeed, inflammatory response regulated by the effect of GC, cannot be terminated in case of GR resistance and diminished sensitivity of immune cells to the hormones (Stark et al., 2001). GR resistance has been observed in patients with MDD (Anacker et al., 2011a, Pariante, 2006). On the other hand, it has been suggested that prolonged inflammation can have a direct effect in reducing GR sensitivity and disrupting its functional properties (Miller et al., 1999).

Taking all together, and having investigated inflammatory response and HPA axis activity, it was of interest to assess the GR to identify any possible alteration in its expression and/or function in CHD patients with and without depression and in order to understand whether or not GR has an involvement in inflammatory activation and HPA axis disturbance in this specific population.

3.4.1 Gene Expression of GR

Along with the expression of genes related to inflammatory cytokines that was demonstrated in section 3.2.2, another candidate gene to investigate was GR gene. As illustrated in Figure 3.17, CHD-D individuals exhibited significantly reduced expression of GR as compared to CHD patients (t=2.105, df=38, **p= 0.042**). Assessing the effect of antidepressants treatment on GR expression did not show any significant result: One way ANOVA, F=2.386, **p=0.106**; CHD-D-noAD vs CHD-D-AD t=0.685 df=14, **p=0.505**. Covariates gender and age also did not have any effect on GR expression outcome; F (1,37)=0.048, **p=0.827** and F(1,37)=995, **p=0.325**, respectively.

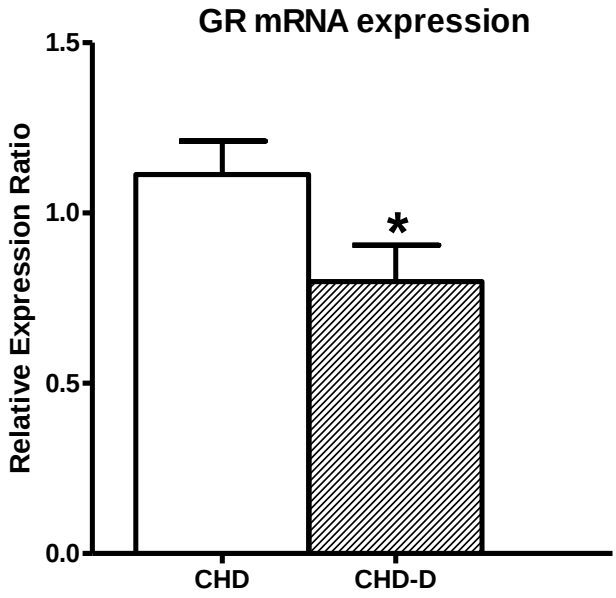


Figure 3.17 GR mRNA expression in CHD patients with and without depression. Data expressed as mean ± SEM, (CHD, n=24; CHD-D, n=16).

CHD depressed patients (n=16) displayed significantly lower GR expression as compared to CHD non-depressed (n=24) (*$p<0.05$).

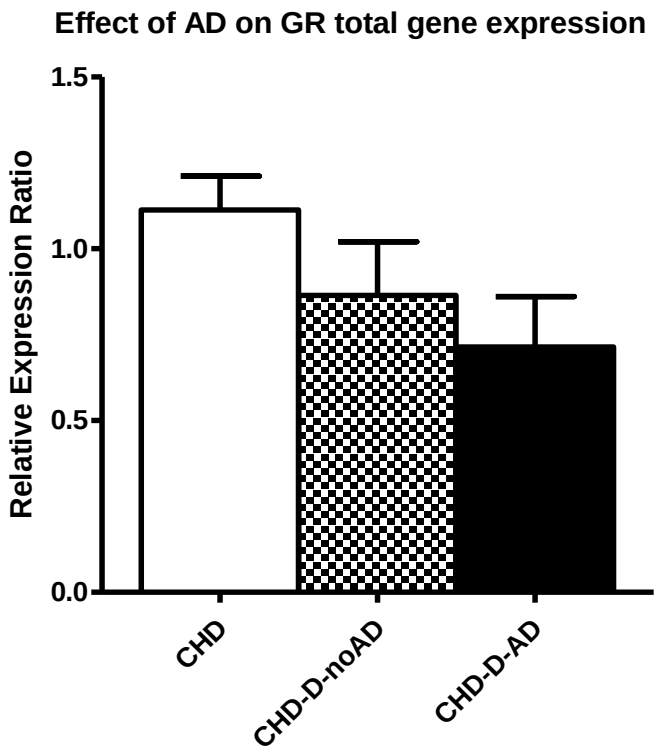


Figure 3.18 The effect of antidepressants on GR total gene expression. Data expressed as mean ± SEM, (CHD, n=24; CHD-D-noAD, n=9; CHD-D-AD, n=7).

Antidepressants treatment had no significant effect on gene expression of GR in CHD patients with depression (CHD-D-AD, n=7) when compared with antidepressant-free CHD depressed individuals (CHD-D-noAD, n=9) ($p>0.05$).

3.4.2 GR Sensitivity

3.4.2.1 Protocol Optimization

As mentioned previously in details (section 2.5), GR function was measured *in vitro* directly on PBMC by culturing them using DEX inhibition of LPS-stimulated IL-6 levels method. The present study optimised and tested the protocol on healthy control before carrying out the experiment on the patients.

Primarily, the cell culture media included CF1 and human serum. However, high levels of IL-6 produced were observed in unstimulated cells. Therefore, additional experiments were designed to understand if any component of the media was triggering the production of IL-6 in unstimulated cells.

As illustrated in Figure 3.19, PBMC were cultured with and without adding human serum in the culture. As well as LPS-simulated cells, unstimulated cells in the presence of human serum produced IL-6. In contrast, cultured PBMC without added human serum in the culture media showed only very low levels of IL-6 when unstimulated, and IL-6 is produced considerably when stimulated by LPS. Therefore, removing human serum from the culture resulted in the expected response and desired outcome.

Another approach was deciding on the number of PBMC sufficient to run the *in vitro* experiment. The initial protocol was using 200K PBMC in each well. It was of interest to investigate the effect of three different drugs on GR sensitivity at the same time of evaluating GR function for each patient. Since there were limited amount of isolated and cryopreserved PBMC available for each patient, the whole experiments would have been only possible if less number of cells could be used. Therefore, the protocol tested four different number of cells; 200, 150, 100, and 50 K.

As demonstrated in Figure 3.20, for all conditions, IL-6 produced by cells shows considerably low levels before stimulation of cells by LPS and remarkably higher production of the cytokine for LPS- simulated cells. In addition, as illustrated in Figure 3.21, for the four different numbers of PBMC, glucocorticoid inhibition of LPS-stimulated IL-6 levels using three different concentration of dexamethasone; 1, 0.03, and 0.001 µM, show the expected dose response curve and similar results for each dexamethasone concentration. Therefore, the decision was made to use 100K PBMC per well in order to investigate GR sensitivity *in vitro* as well as the effect of antidepressants clomipramine and citalopram, and omega-3 on GR function at the same time.

Finally, the protocol was examined on 5 healthy control samples. As presented in Figure 3.22, PBMC showed remarkably increased in IL-6 levels after being stimulated by LPS. In addition, dexamethasone inhibition of LPS-stimulated IL-6 production is illustrated in Figure 3.23 testing five different concentration of dexamethasone; 1, 0.1, 0.03, 0.01, and 0.001 µM. The figure represents the expected dose dependent response to the effect of the glucocorticoid mediated by GR that shows the higher dexamethasone concentration the less IL-6 were produced.

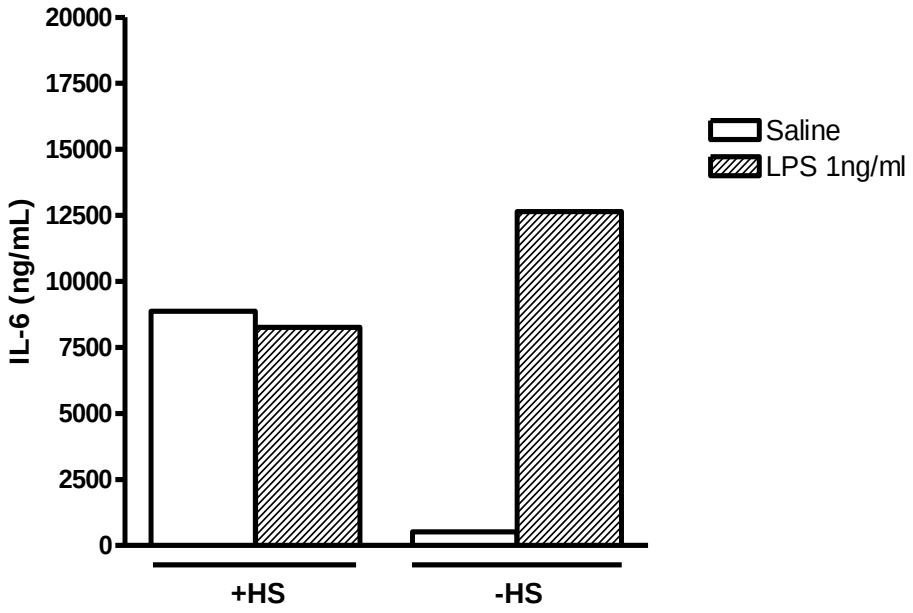


Figure 3.19 IL-6 levels (ng/ml) of PBMC before and after in vitro LPS stimulation culturing the cells with and without human serum.

In vitro stimulation of PBMC by LPS (1 ng/ml) with and without human serum (HS) showed that adding HS in the culture (+HS) triggered unstimulated cells (white column) to produce IL-6. When PBMC were stimulated by LPS and without adding HS (-HS) in the culture, unstimulated cells produced very low levels of IL-6, while the levels increased remarkably after stimulation (hashed column).

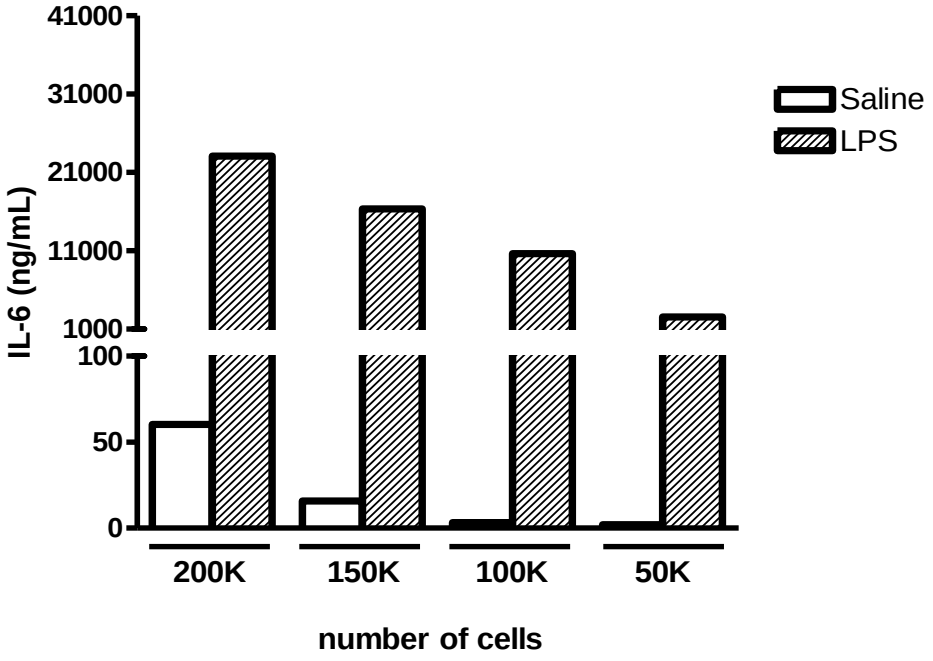


Figure 3.20 IL-6 levels (ng/ml) of PBMC before and after *in vitro* LPS stimulation culturing different number of cells: 200, 150, 100, and 50 K.

The graph demonstrates four different PBMC cultures each containing specific number of cells, 200, 150, 100, and 50 K. For all conditions, IL-6 produced (ng/ml) by cells showed considerably low levels before stimulation of cells by LPS (white columns) and remarkably higher production of the cytokine for LPS simulated cells (hashed columns).

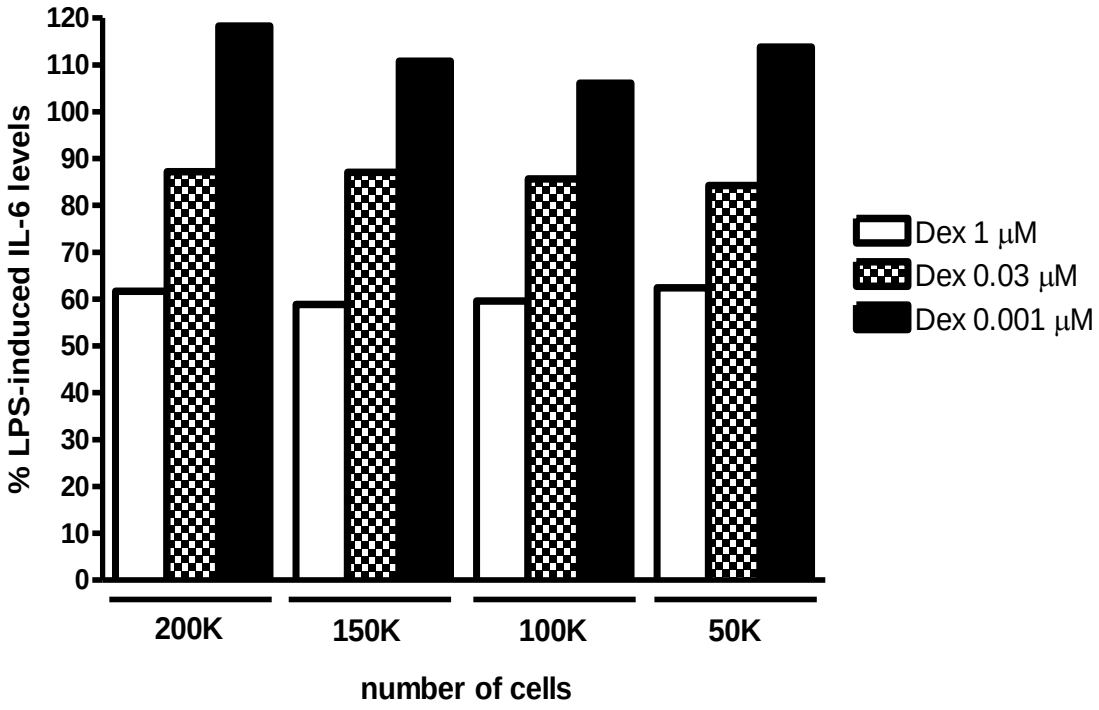


Figure 3.21 Dexamethasone inhibition of LPS stimulated IL-6 production in PBMC culturing different number of cells: 200, 150, 100, and 50 K.

Dexamethasone (1, 0.03, and 0.001 μM) inhibition of LPS-stimulated IL-6 levels showed similar results when culturing four different numbers of cells: 200, 150, 100, and 50 K. Results are expressed by the percent dexamethasone inhibition (LPS-stimulated IL-6 levels with dexamethasone divided by LPS-stimulated IL-6 levels without dexamethasone).

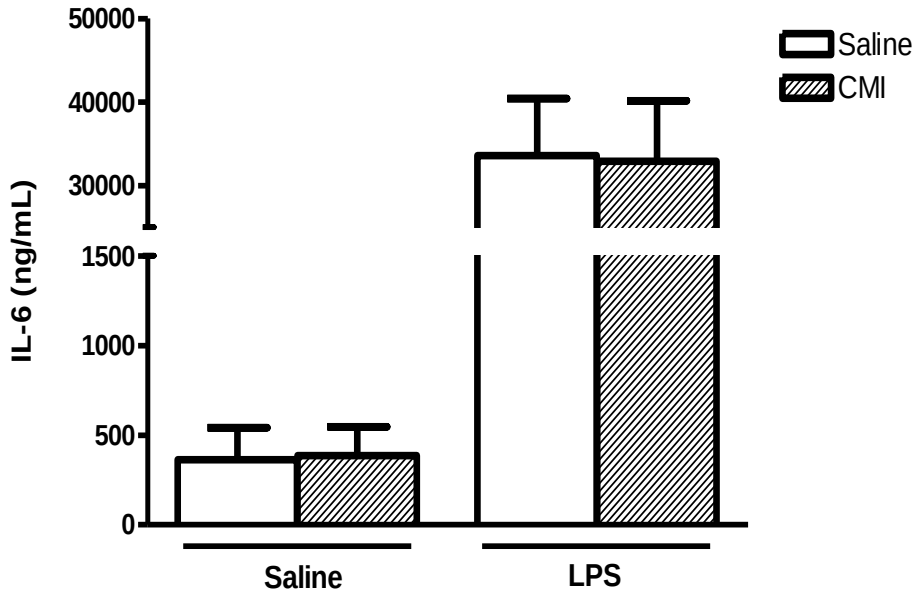


Figure 3.22 IL-6 levels (ng/ml) of PBMC before and after *in vitro* LPS stimulation with and without clomipramine in healthy individuals. Data expressed as mean ± SEM, (n=5).

The graph represents IL-6 production (ng/mL) before and after stimulation of PBMC by LPS with (hashed columns) and without (white columns) clomipramine (10 μM) in healthy controls (n=5).

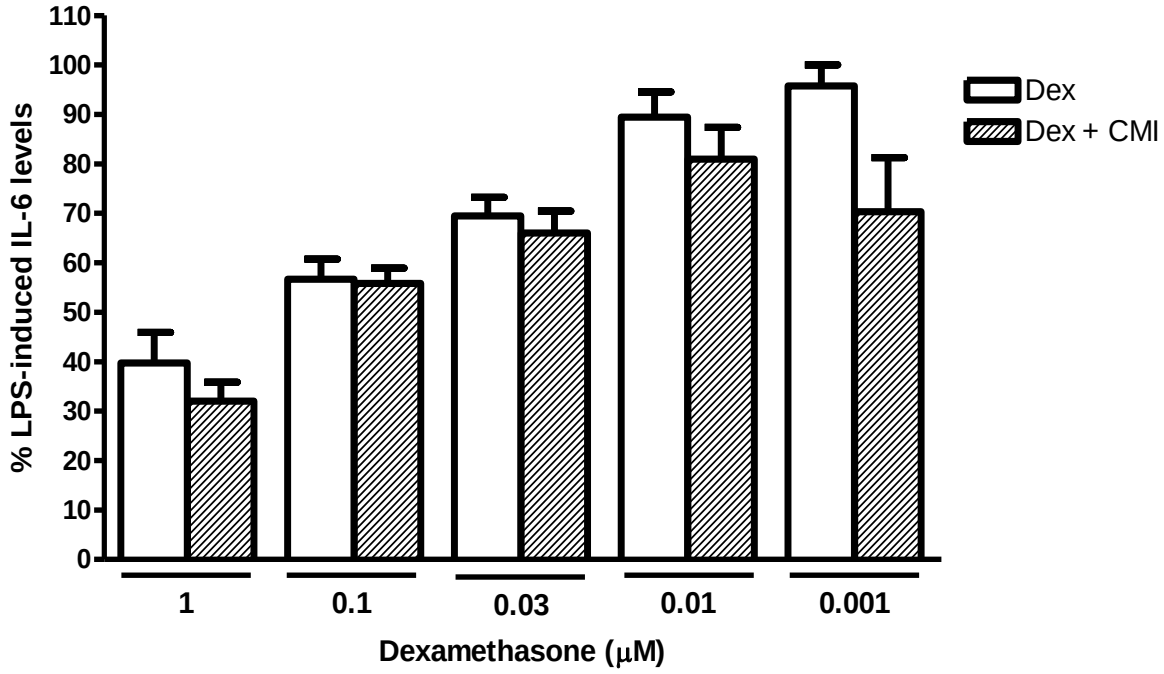


Figure 3.23 Dexamethasone inhibition of LPS stimulated IL-6 production in PBMC of healthy individuals with and without clomipramine. Data expressed as mean ± SEM, (n=5).

Dexamethasone (1, 0.1, 0.03, 0.01, and 0.001 μM) inhibition of LPS-stimulated IL-6 levels in PBMC of healthy controls (n=5) showed expected dose dependent response to the effect of the glucocorticoids. Results are expressed by mean ± SEM of the percent dexamethasone inhibition (LPS-stimulated IL-6 levels with dexamethasone divided by LPS-stimulated IL-6 levels without dexamethasone).

3.4.2.2 *In Vitro* Stimulation of PBMC in CHD Patients with and without Depression

In this study an *in vitro* evaluation of GR function was performed on previously cryopreserved PBMC stored in liquid nitrogen. Utilization of cryopreserved PBMC for immunologic assays in clinical trials and studies has been recently widely employed to allow collecting required samples from all patients particularly when large numbers of samples are required, and to perform the assays together in a specific period of time and in a single laboratory. These reasons would allow us to eliminate or at least minimize inter-assay variation and inter-laboratory variability that have been reported by studies as confounders (Chen et al., 2010, Shearer et al., 2003). The advantage of using frozen PBMC in this PhD study was that GR function could be performed in parallel for depressed and non-depressed patients.

As described in details before in section 2.5.2, the recovery and viability of PBMC following thawing were examined (>95%) to ensure reliable results in performing GR functional assay. Monitoring the quality of the cryopreserved PBMC and obtaining a viability of >80% has been suggested as an important requirement in immunological assays (Dyer et al., 2007), since changes in cells viability have been reported as a potential challenge posing from cryopreservation of PBMC (Jeurink et al., 2008, Koch et al., 1991).

Using bacterial LPS to stimulate PBMC for the production of cytokines has been extensively used in *in vitro* studies mimicking the inflammatory condition. As illustrated in Figure 3.24, in the absence of LPS, PBMC display considerably low levels of IL-6 detected. Following stimulation of PBMC by LPS, IL-6 is produced significantly as shown by remarkably high levels of the cytokine in both CHD patients with and without

depression. The IL-6 released following LPS stimulation did not differ between the two groups (U=67.00, **p=0.312**).

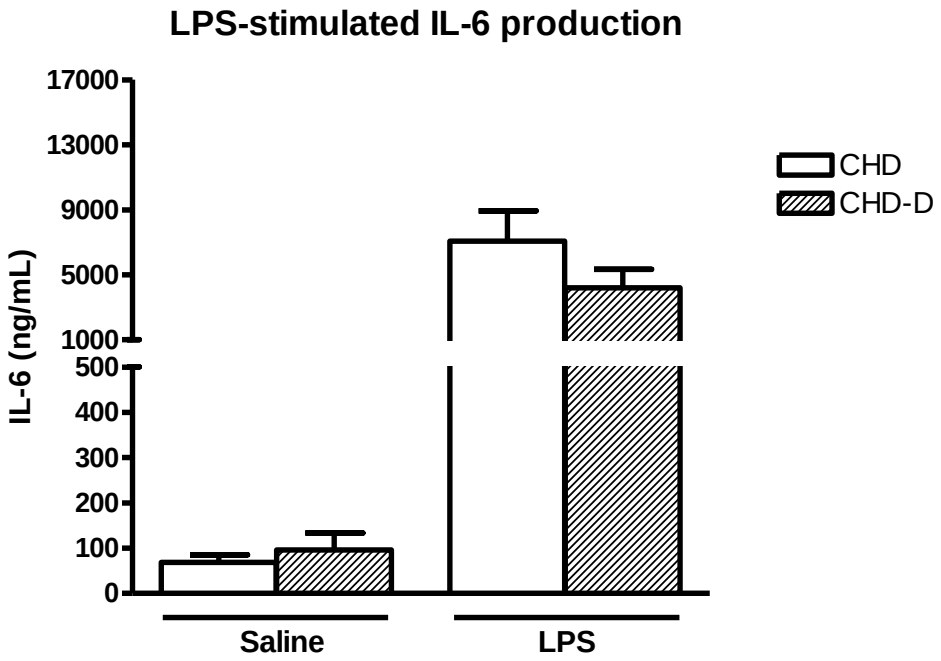


Figure 3.24 IL-6 levels (ng/mL) of PBMC before and after in vitro LPS stimulation in CHD patients with and without depression. Data expressed as mean ± SEM, (CHD, n=16; CHD-D, n=12).

In vitro stimulation of PBMC (100K) by LPS (1 ng/mL) induced production of IL-6 (ng/mL) in CHD patients with (hashed columns, n=12) and without (white columns, n=16) depression. The levels of IL-6 were low in the absence of LPS in both groups followed by a considerable increased upon LPS-stimulation.

3.4.2.3 Dexamethasone Inhibition of LPS-induced IL-6 Production of PBMC

Performing *in vitro* method of synthetic glucocorticoid dexamethasone inhibition of LPS-stimulated IL-6 production in PBMC, GC mediated GR function was assessed in CHD patients with and without depression. In this method, the higher concentration of glucocorticoid should induce the greater inhibition of LPS-stimulated IL-6 levels.

As illustrated in Figure 3.25, both CHD patients with and without depression showed dose response curve and suppression of LPS-stimulated IL-6 levels in a concentration dependent manner. However, compared to non-depressed group, CHD depressed individuals exhibited higher levels of IL-6 for each concentration of dexamethasone that indicated lower ability of GR to respond to the inhibition effect of the glucocorticoid on LPS-stimulated IL-6 production (Two-way ANOVA, F=12.26, **p=0.0006**).

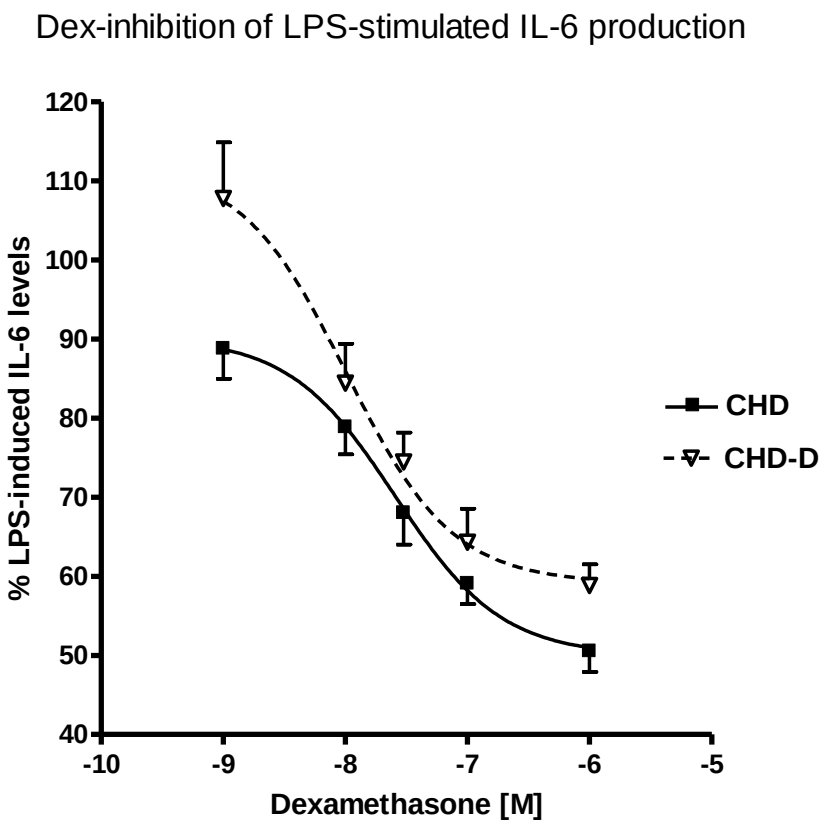


Figure 3.25 Dexamethasone inhibition of LPS stimulated IL-6 production in PBMC of CHD patients with and without depression. Data expressed as mean ± SEM, (CHD, n=16; CHD-D, n=12).

As compared to CHD non-depressed individuals (n=16) represented by solid line, CHD depressed patients (n=12) indicated by dotted lines have lower GR sensitivity ($p<0.001$). In both groups, introducing higher concentration of dexamethasone from 10^{-9} to 10^{-6} [M] is found less production of IL-6, and CHD depressed showing higher levels of IL-6 produced for each concentration of the glucocorticoid suggesting less GR sensitivity to the inhibition effect of dexamethasone. Results are expressed by mean ± SEM of the percent glucocorticoid inhibition (LPS-stimulated IL-6 levels with dexamethasone divided by LPS-stimulated IL-6 levels without dexamethasone).

3.4.2.4 Half Maximal Inhibitory Concentration of Dexamethasone (IC50-DEX)

Effectiveness of dexamethasone to inhibit production of *in vitro* LPS-stimulated IL-6 that is mediated by GR was measured by calculating half maximal inhibitory concentration (50% IC or IC50). IC50 values derived from DEX dose response curve using GraphPad Prism software.

As illustrated in Figure 3.26, CHD depressed patients exhibited significantly higher IC50 as compared to CHD non-depressed individuals; IC50-DEX [M] (mean ± SEM); CHD 7.6 ± 0.1, CHD-D 8.1 ± 0.2, t=3.267, df=26, **p=0.0031**. The results showed that depressed group required a higher concentration of dexamethasone at which 50% of IL-6 synthesis is inhibited which confirmed a reduced ability of GR to respond to glucocorticoids.

In addition, as demonstrated in Figure 3.27, IC50-DEX was found to be significantly associated with the BDI scores in CHD patients with and without depression (r=.505, **p=.006**, n=28) indicating the relation between GR resistance and the severity of depressive symptomatology in this population.

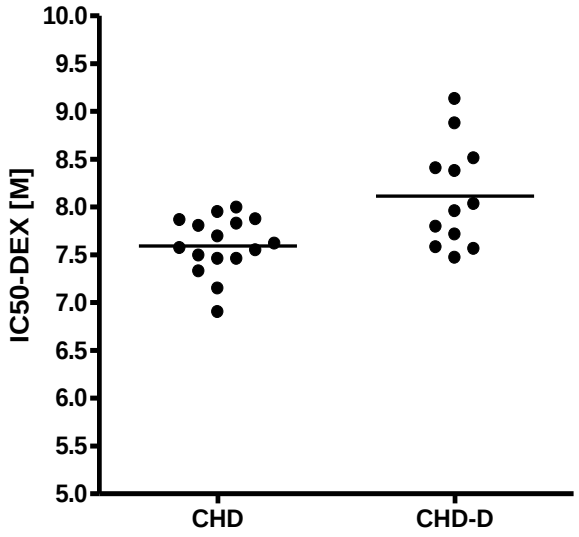


Figure 3.26 IC50-DEX [M] for LPS stimulated IL-6 production in PBMC of individual CHD patients with and without depression. (CHD, n=16; CHD-D, n=12).

Calculating IC50-DEX [M] of individual subjects showed CHD depressed patients (CHD-D, n=12) required significantly higher concentration of dexamethasone for the half inhibitory effect on LPS-stimulated IL-6 production as compared to CHD non-depressed (CHD, n=16) ($p<0.01$). The dots represent the IC50, and the solid lines demonstrate the mean of the values.

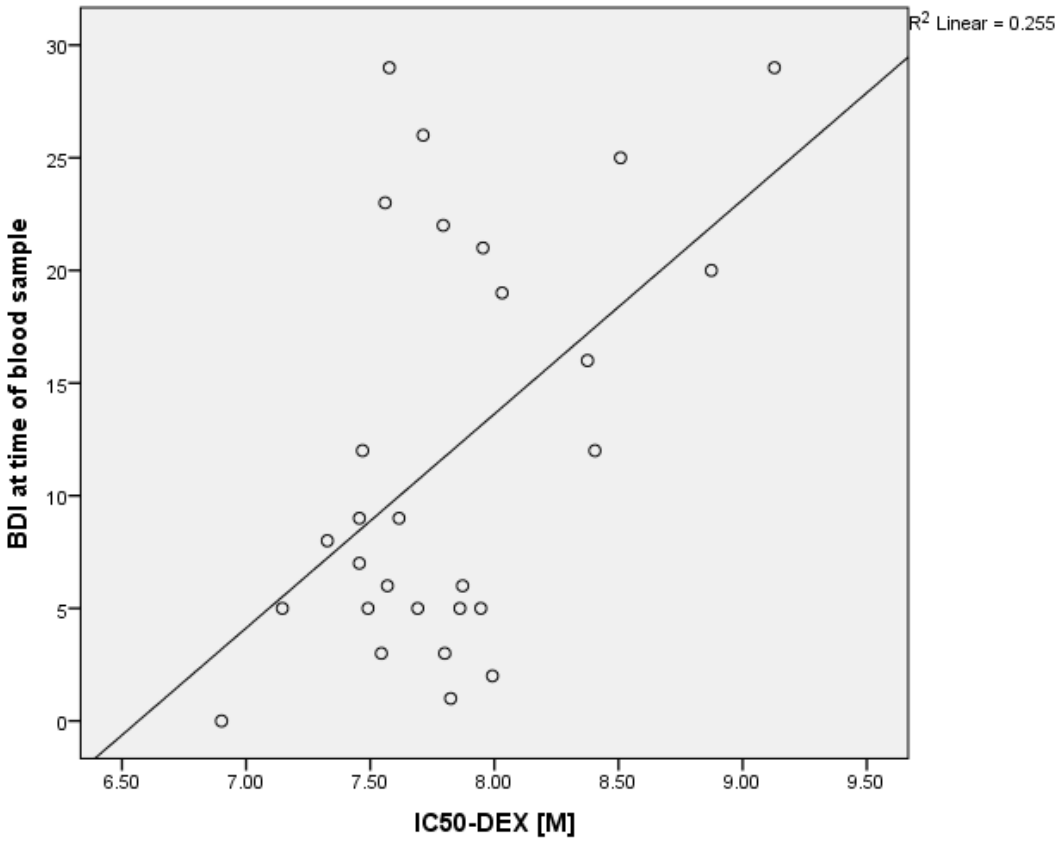


Figure 3.27 Association of IC50-DEX [M] with BDI scores in CHD patients with and without depression (r=.505, **p<.01, n=28).

IC50-DEX [M] was significantly associated with the BDI scores in CHD patients with and without depression (r=.505, **p<.01, n=28). The positive correlation indicates that GR resistance is associated with the severity of depressive symptoms in these patients.

3.4.3 Effect of Drugs on GR Sensitivity

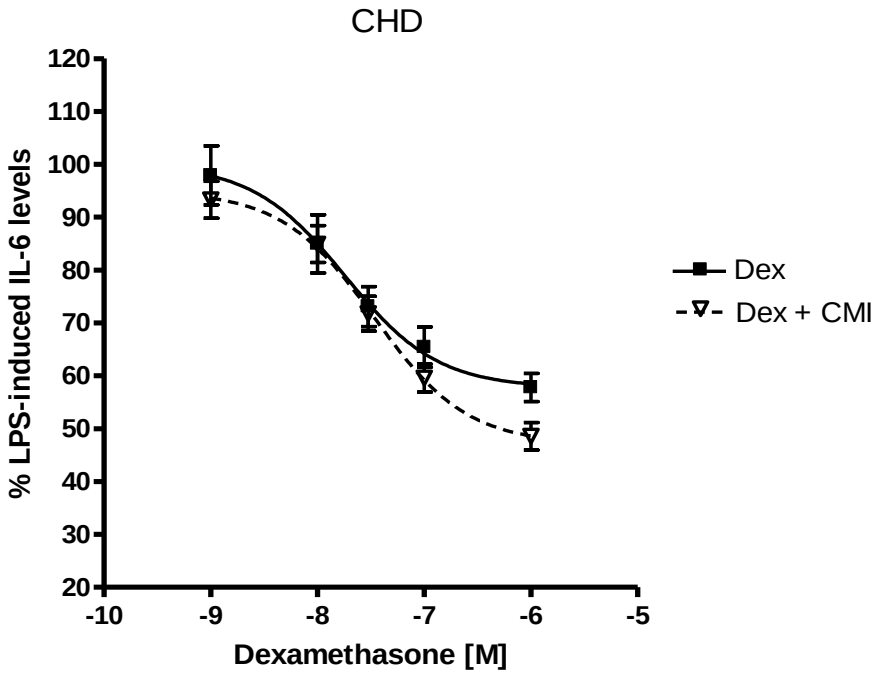
Assessing GR function *in vitro*, the effect of two antidepressants clomipramine (TCA) and citalopram (SSRI) as well as omega-3 PUFA as an agent with anti-inflammatory and antidepressive properties was also investigated on GR sensitivity in this PhD thesis, in order to understand whether these medications exert any effect on reversing the GR resistance in these patients.

3.4.3.1 Effect of Clomipramine on GR Sensitivity

The tricyclic antidepressants are suggested to have the ability to modulate GR function (McQuade and Young, 2000). This class of antidepressants has been shown to enhance GR function (Pariante et al., 1997) and contribute in regulation of intracellular cortisol concentration and GR expression (Pariante et al., 2003). Most of the studies on the effect of these antidepressants has been done in cell line and animal models, and very few in a clinical population including the study by Carvalho that will be discussed later (Carvalho et al., 2008). Therefore, it was of interest to investigate the effect of clomipramine in this class on GR function *in vitro* in this clinical setting.

As demonstrated in Figure 3.28, clomipramine improved the sensitivity of GR in CHD depressed patients as shown by the reduction in the levels of LPS stimulated IL-6 production for each concentration of dexamethasone ($F=8.835$, $p= 0.0036$). However, the significant effect was not observed in GR sensitivity in CHD patients without depression ($F= 3.021$, $p= 0.0840$).

a)



b)

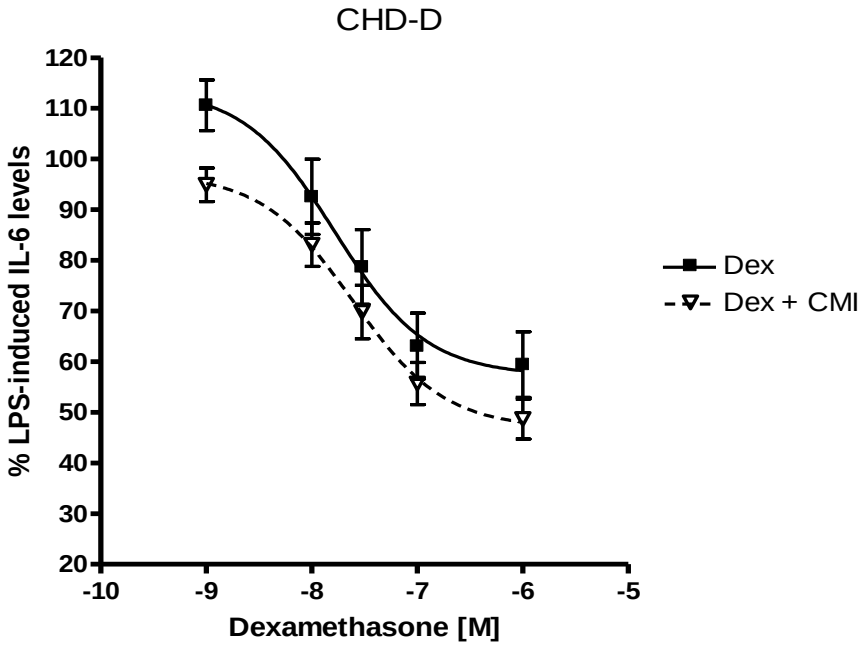


Figure 3.28 Effect of clomipramine (10 µM) *in vitro* on GR mediated dexamethasone inhibition of LPS stimulated IL-6 production in PBMC of CHD patients with (b) and without (a) depression. Data expressed as mean ± SEM, (CHD, n=15; CHD-D, n=10).

In CHD patients b) with depression (n=10), in the presence of clomipramine (10 µM) (dotted lines); LPS stimulated IL-6 levels were decreased for each concentration of

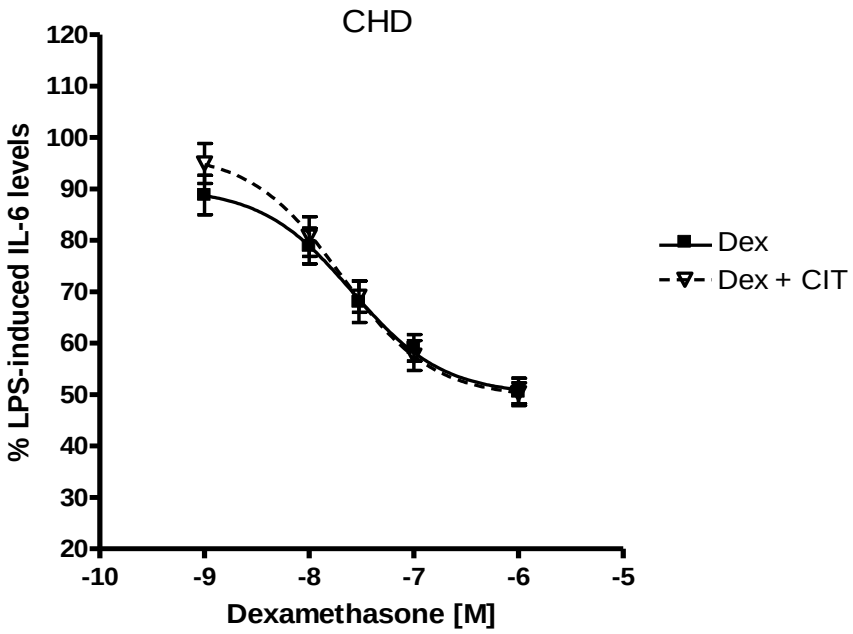
dexamethasone. Therefore, clomipramine increased GR sensitivity in CHD depressed patients ($p<0.01$). However, clomipramine *in vitro* did not have an effect on GR sensitivity in CHD non-depressed individuals ($n=15$) ($p>0.05$). Results are expressed by mean ± SEM of the percent glucocorticoid inhibition (LPS-stimulated IL-6 levels with glucocorticoid divided by LPS-stimulated IL-6 levels without glucocorticoids).

3.4.3.2 Effect of Citalopram on GR Sensitivity

The choice of antidepressants for CHD patients requires special attention since epidemiological studies reveal the use of antidepressants could be associated with both decreased and increased cardiac risk (Sherwood et al., 2007). For example TCAs and MAOIs are known to have cardiotoxic side effects, and therefore, not a preferable choice for treatment of depression in heart disease patients. The SSRI antidepressants and specially citalopram and sertraline are recognised to be safe and effective, thus the first-line antidepressants for CHD patients who suffer from moderate to severe as well as recurrent depression (Glassman et al., 1993, Glassman et al., 2002, Lespérance et al., 2007).

As part of this PhD thesis, it was of relevance to investigate whether or not citalopram has any effect on GR function *in vitro*. The result presented in Figure 3.29, revealed no significant effect of citalopram in GR sensitivity in CHD patients without depression (CHD F= 0.4726, p=0.4927). However, in CHD depressed patients who already exhibited decreased GR sensitivity (Figure 3.25 and Figure 3.26), citalopram appeared to significantly improve the sensitivity of GR (CHD-D F=14.83, p=0.0002).

a)



b)

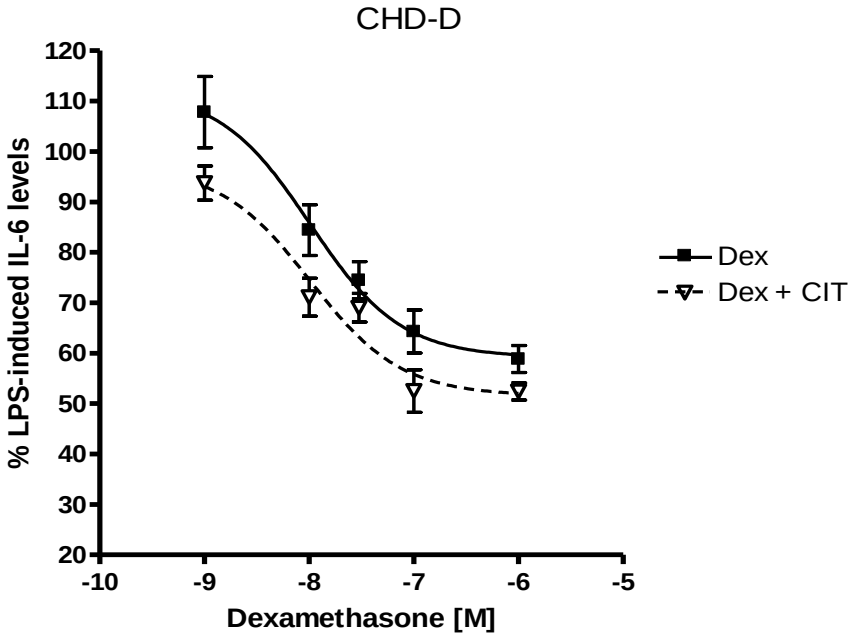


Figure 3.29 Effect of citalopram (10 µM) in vitro on GR mediated dexamethasone inhibition of LPS stimulated IL-6 production in PBMC of CHD patients with (b) and without (a) depression. Data expressed as mean ± SEM, (CHD, n=15; CHD-D, n=10).

In CHD patients b) with depression (n=10), in the presence of citalopram (10 µM) (dotted lines); LPS stimulated IL-6 levels were decreased for each concentration of

dexamethasone. Therefore, citalopram increased GR sensitivity in CHD depressed patients ($p<0.001$). However, citalopram *in vitro* did not have any effect on GR sensitivity in CHD non-depressed individuals ($n=15$) ($p>0.05$). Results are expressed by mean ± SEM of the percent glucocorticoid inhibition (LPS-stimulated IL-6 levels with glucocorticoid divided by LPS-stimulated IL-6 levels without glucocorticoids).

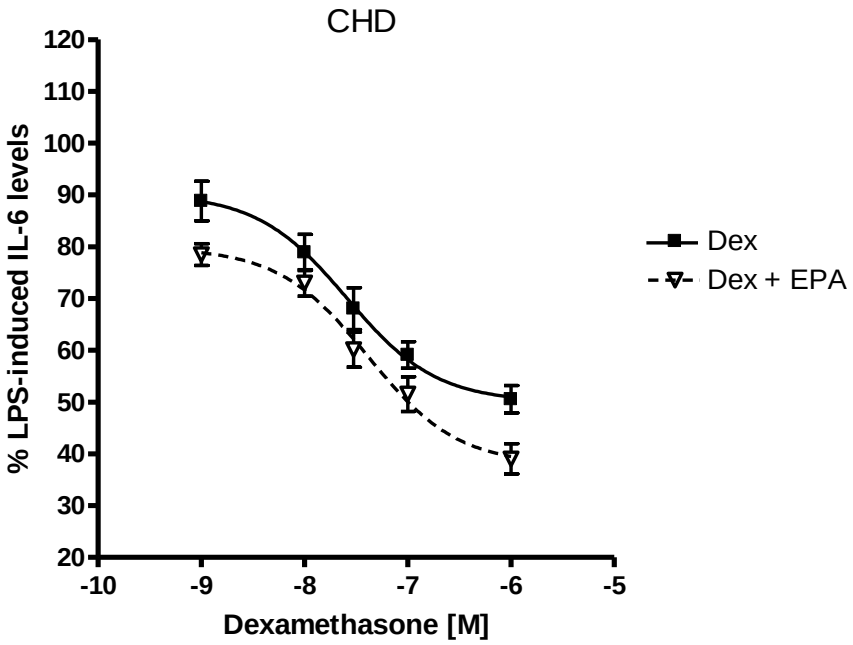
3.4.3.3 Effect of EPA on GR Sensitivity

Omega-3 fatty acids have been suggested as a therapeutic option in patients with heart disease (Christensen et al., 1996). Dietary omega-3 has a protective effect on adverse cardiac events specially heart attack according to the epidemiological studies (Breslow, 2006, Harris et al., 2007, Lee et al., 2008). On the other hand, the association of this medication with depression has been described in section 1.2.9. However, the mechanism of their action has not been completely understood.

Eicosapentaenoic acid (EPA) derived from fish oil is the biologically potent omega-3 PUFA that is widely used in animal and clinical studies (Simopoulos, 2002) . Thus this PhD thesis also investigated the effect of omega-3 specifically on GR function as this might throw more light on their specific mode of action as anti-inflammatory and antidepressant medication.

As illustrated in Figure 3.30, GR function in CHD patients with and without depression was assessed *in vitro* in the presence of EPA. The result showed EPA increased GR sensitivity in CHD patients with depression ($F= 27.98$, $p<0.0001$) as well as in CHD non-depressed individuals ($F=18.71$, $p<0.0001$) as presented by decreased production of LPS stimulated IL-6 for each concentration of dexamethasone.

a)



b)

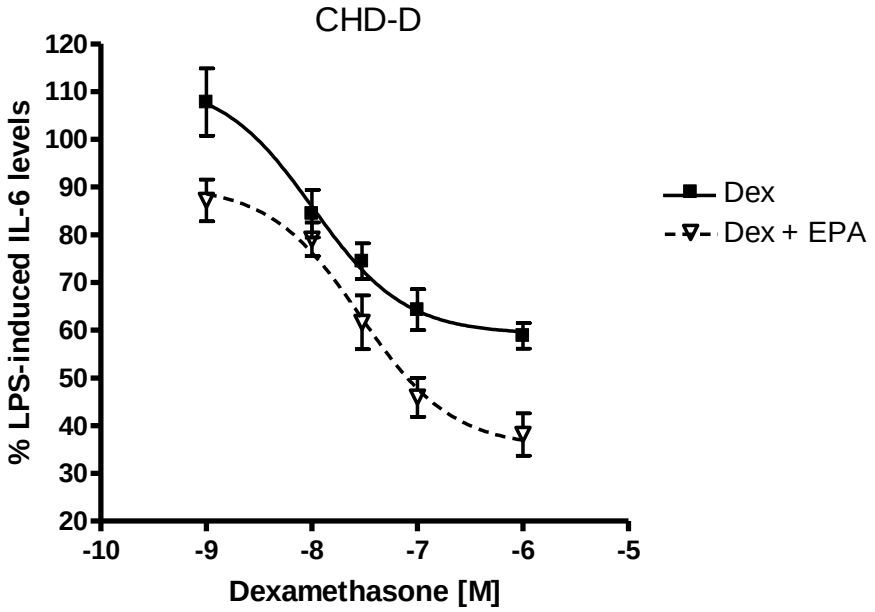


Figure 3.30 Effect of EPA (10 µM) in vitro on GR mediated dexamethasone inhibition of LPS stimulated IL-6 production in PBMC of CHD patients with (b) and without (a) depression. Data expressed as mean ± SEM, (CHD, n=15; CHD-D, n=10).

For both CHD patients b) with (n=10) and a) without (n=15) depression, in the presence of EPA (10 µM) (dotted lines), LPS stimulated IL-6 levels were decreased for each

concentration of dexamethasone. Therefore, EPA increased GR sensitivity in CHD patients with and without depression ($p<0.0001$). Results are expressed by mean ± SEM of the percent glucocorticoid inhibition (LPS-stimulated IL-6 levels with glucocorticoid divided by LPS-stimulated IL-6 levels without glucocorticoids).

3.5 Kynurenine Pathway of Tryptophan Metabolism in CHD Patients with and without Depression

The kynurenine pathway of the tryptophan metabolism and its relation to inflammation and depression has been described in details in 1.2.7. Considering CHD as an inflammatory condition, it was justified to investigate this system in the present population of CHD patients with and without comorbid depression. Therefore, tryptophan metabolism pathway was assessed with a focus on the kynurenine diversion of the system, and measuring the circulatory levels of the metabolites produced.

As presented in the Table 3.5, CHD patients with depression showed lower serum levels of tryptophan when compared with CHD non-depressed individuals: (mean ± SEM); CHD 12.64 ± 0.64 µg/ml, CHD-D 10.42 ± 0.58 µg/ml, t=2.247, df=43, **p=0.030**. Kynurenine/tryptophan (KYN/TRP) ratio, indicator of activated IDO, were found significantly higher in depressed CHD compered to non-depressed group (mean ± SEM); CHD 62.21 ± 3.10, CHD-D 74.43 ± 5.26, t=-2.144, df=43, **p=0.038**. The serum levels of kynurenic acid, 3-HK and 3-HAA did not differ in CHD patients with and without depression (p>0.05). As illustrated in Figure 3.31, KYN/TRP ratio were found to be associated with the levels of the neurotoxic metabolite of the kynurenine pathway 3-HK in CHD patients with and without depression (r_s =.405, **p=.006**, n=45), coincided with no association between KYN/TRP ratio and the levels of the neuroprotective metabolite kynurenic acid in these patients (r =.096, **p=.53**, n=45).

Having investigated the inflammatory response (section 3.2.1), the kynurenine pathway was also assessed in relation to inflammation. As presented before in Figure 3.1, CHD patients with and without depression showed high levels of inflammation as measured by

circulatory CRP levels. CRP as an inflammatory biomarker were found to be associated with KYN/TRP ratio (r=.322, **p=.040**, n=41) as well as with the neurotoxic metabolite 3-HK (r_s =.420, **p=.006**, n=41) in CHD patients with and without comorbid depression.

Table 3.5 Serum levels of kynurenine pathway metabolites in CHD patients with and without depression.

	CHD (n=29) (mean ± SEM)	**CHD-D (n=16) (mean ± SEM)**	**Test and significance**
TRP (µg/ml)	12.64 ± 0.64	10.42 ± 0.58	t=2.25, **p=0.030***
KYN (ng/ml)	843.50 ± 54.37	763.85 ± 43.18	U=209.0, z= -0.55, p=0.59
KYN/TRP	62.21 ± 3.10	74.43 ± 5.26	t=-2.14, **p=0.038***
KYNA (ng/ml)	15.57 ± 1.18	13.21 ± 1.79	t= 1.14, p=0.259
3-HK (ng/ml)	18.85 ± 1.77	18.73 ± 1.43	U=199.5, z= -0.77, p=0.44
3-HAA (ng/ml)	34.31 ± 3.35	33.50 ± 5.13	U=219.0, z= -0.31, p=0.76

TRP, tryptophan; KYN, Kynurenine; KYNA, Kynurenic acid; 3-HK, 3-hydroxykynurenin; 3-HAA, 3-hydroxyanthranilic acid

As compared to CHD non-depressed individuals (n=29), CHD depressed patients (n=16) exhibited significantly decreased in serum TRP levels (µg/ml) lower GR (*$p<0.05$). KYN/TRP ratio was significantly higher in depressed individuals when compared with non-depressed group ($p<0.05$). The serum levels of KYN, KYNA, 3-HK and 3-HAA did not differ in CHD patients with and without depression ($p>0.05$).

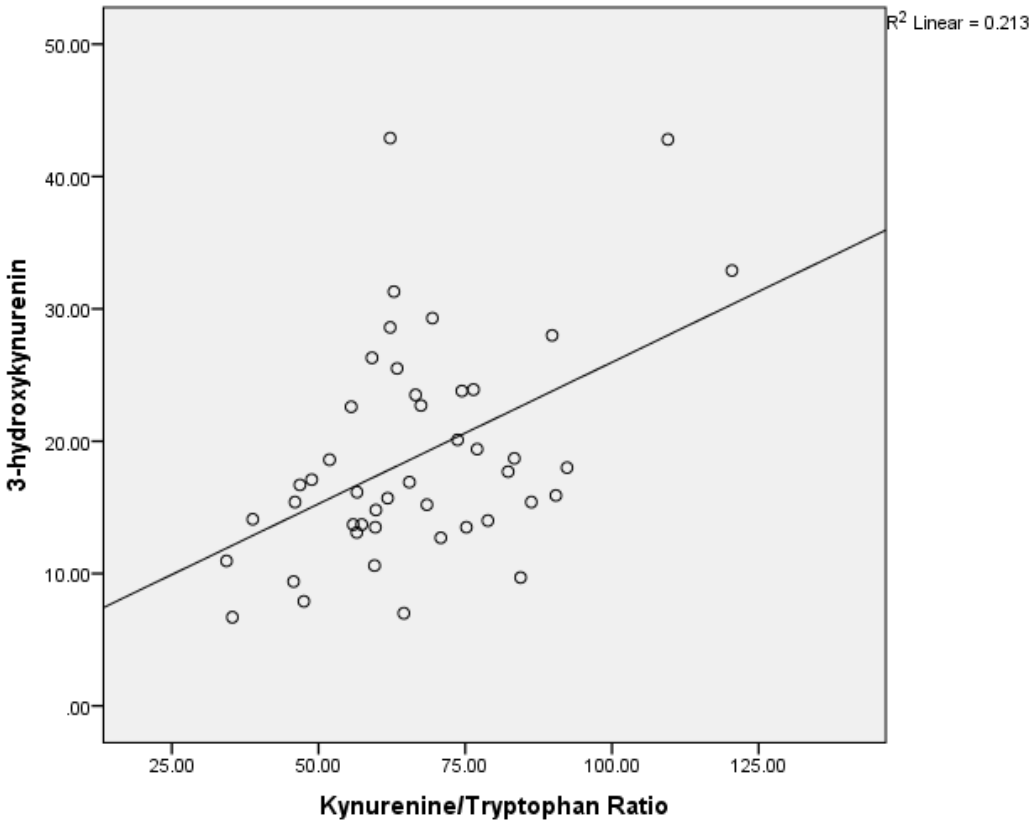


Figure 3.31 Positive correlation of KYN/TRP ratio with 3-HK in CHD patients with and without depression (r_s=.405, **p<.01, n=45).

Kynurenine/Tryptophan (KYN/TRP) ratio was significantly associated with the levels of 3-HK in CHD patients with and without depression (r_s=.405, **p<.01, n=45). The positive correlation indicates that greater rates of IDO enzyme activity (as measured by the KYN/TRP ratio) were associated with higher production of 3-HK, the neurotoxic metabolite of the kynurenine pathway.

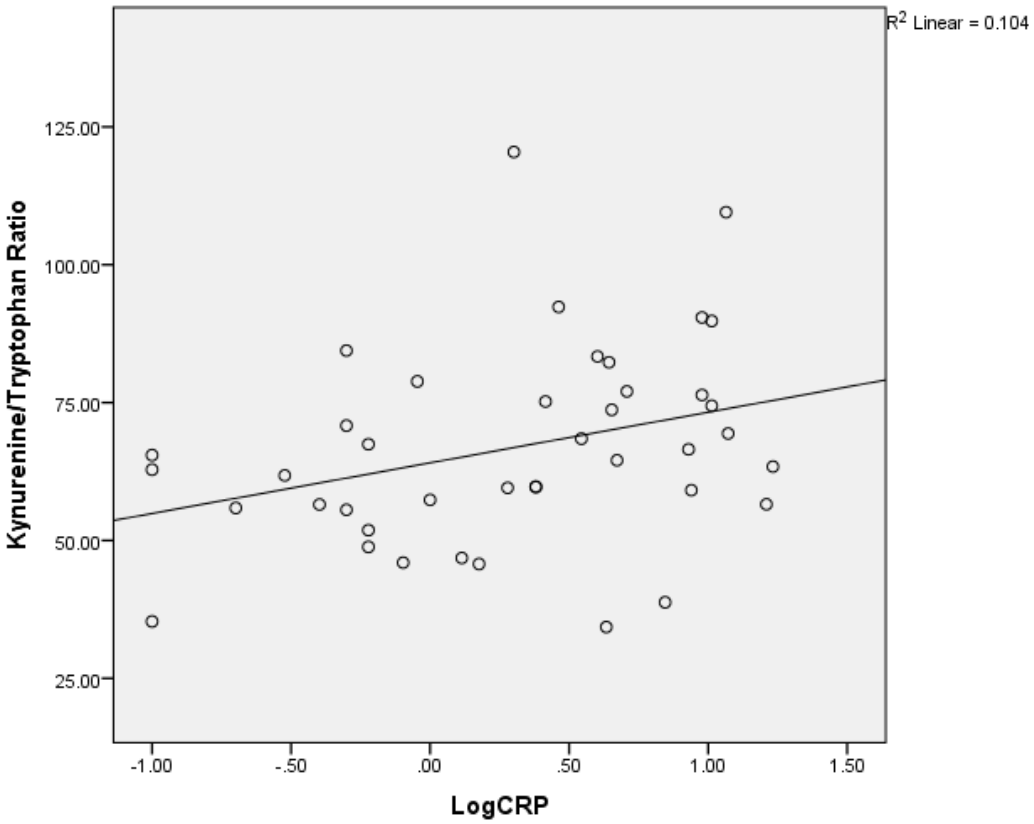


Figure 3.32 Association between circulatory CRP and KYN/TRP ratio in CHD patients with and without depression (r=.322, *p<.05, n=41).

CRP was significantly associated with KYN/TRY ratio in CHD patients with and without depression (r=.322, *p<.05, n=41). The positive correlation indicated that higher inflammation in these patients was associated with greater rate of IDO activity as measured by the KYN/TRY ratio.

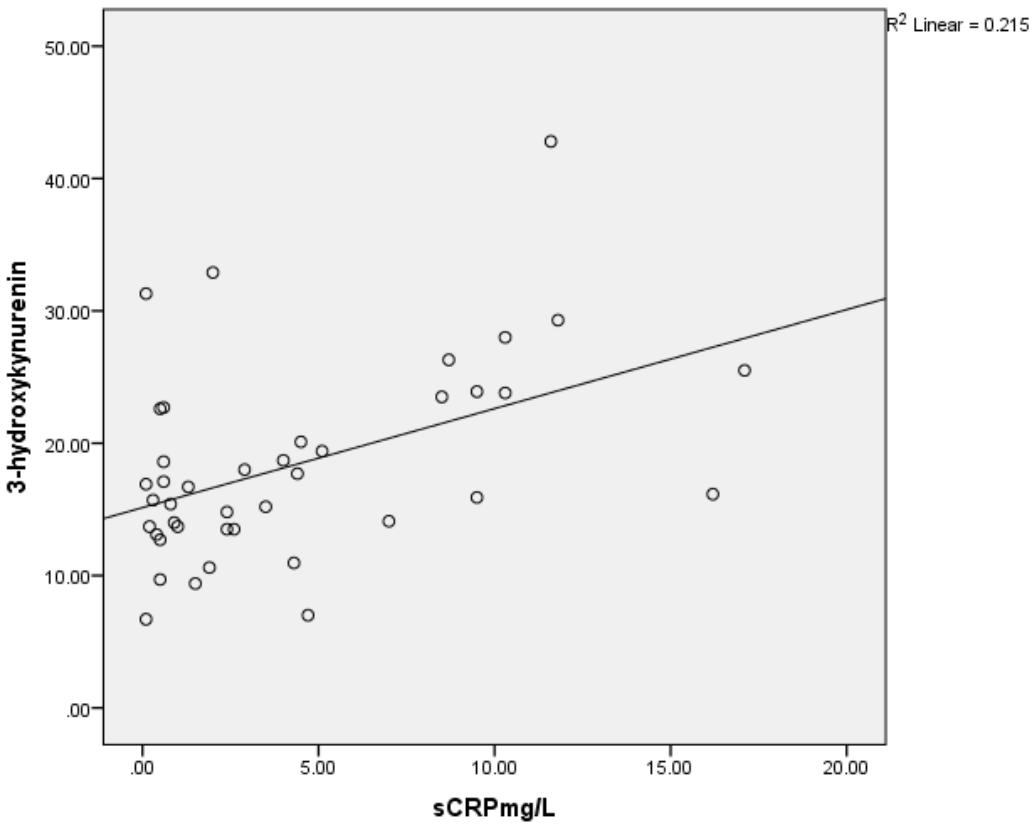


Figure 3.33 Association between circulatory CRP and 3-HK in CHD patients with and without depression (r_s=.420, **p<.01, n=41).

CRP was significantly associated with 3-HK in CHD patients with and without depression (r_s=.420, **p<.01, n=41). The positive correlation indicated that higher inflammation in these patients was associated with higher production of 3-HK, the neurotoxic metabolite of the kynurenine pathway.

3.6 Plasma VEGF in CHD Patients with and without Depression

Originally identified for its ability to promote vascular permeability (Senger et al., 1986), vascular endothelial growth factor (VEGF) is known as a secreted angiogenic mitogen (Leung et al., 1989). It is the most potent growth factor for endothelial cells and a main regulator of angiogenesis and vasculogenesis (Byrne et al., 2005, Keck et al., 1989) as well as one of the key factors involved in BBB stability (Lee et al., 2003). Apart from its involvement in endothelial processes, VEGF exhibit a neurologic role involved in neurogenesis and exerts neuroprotective effect (Jin et al., 2002, Storkebaum et al., 2004).

Due to the crucial involvement in vascular system as well as neurological processes, VEGF was found to be an interesting biological candidate to be investigated in CHD patients with and without depression.

Following measuring the VEGF levels, the obtained values did not meet the parametric assumptions being not normally distributed, thus the data were log transformed to improve suitability for parametric statistics. Comparing the groups showed that CHD depressed patients exhibited significantly higher plasma VEGF levels than CHD non-depressed: Log VEGF (mean ± SEM); CHD 1.816 ± 0.07, CHD-D 2.06 ± 0.09, $t(48)=-2.251$, **p=0.029**. However, for presentation purposes, the raw values were used as illustrated in Figure 3.34.

An increased in VEGF levels in CHD depressed patients appeared to be affected by antidepressant treatment. Although, the result were not found statistically significant, they showed a strong trend for elevation in VEGF levels in CHD depressed who were on antidepressants compared to antidepressant-free CHD depressed and CHD non-depressed groups; One-way ANOVA: Log VEGF $F(2,47)= 3.121$, **p=0.0534**; CHD vs CHD-D-

noAD t(40)=1.48, **p= 0.147**; CHD vs CHD-D-AD t(35)=2.24, **p=0.031**; CHD-D-noAD vs CHD-D-AD t(19)=1.048, **p=0.308**.

Gender and age were analysed as covariates and no significant effect was found on the VEGF outcome: F(1,47)=0.669, **p=0.417** and F(1,47)=0.327, **p=0.570**, respectively.

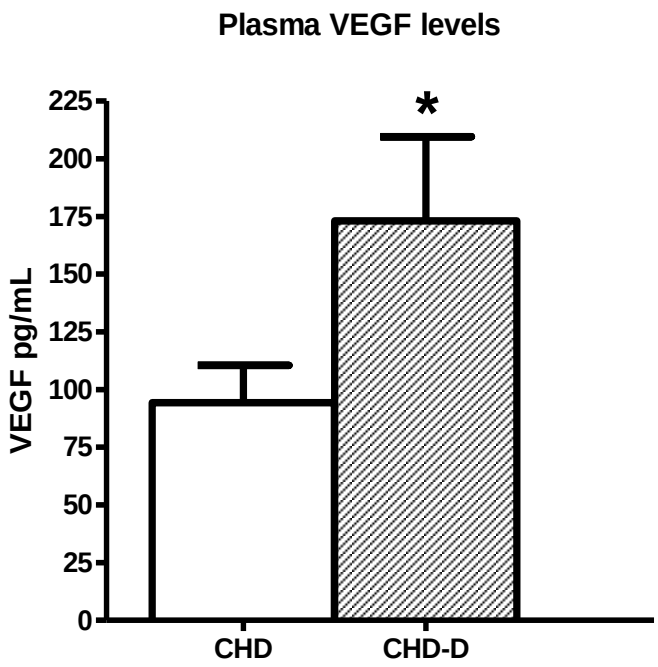


Figure 3.34 Plasma VEGF levels (pg/mL) in CHD patients with and without depression Data expressed as mean ± SEM, (CHD, n=29; CHD-D, n=21).

Compared to CHD patients without depression (CHD, n=29), depressed CHD individuals (CHD-D, n=21) showed significantly higher levels of circulatory VEGF (pg/mL) as measured in plasma (*$p<0.05$).

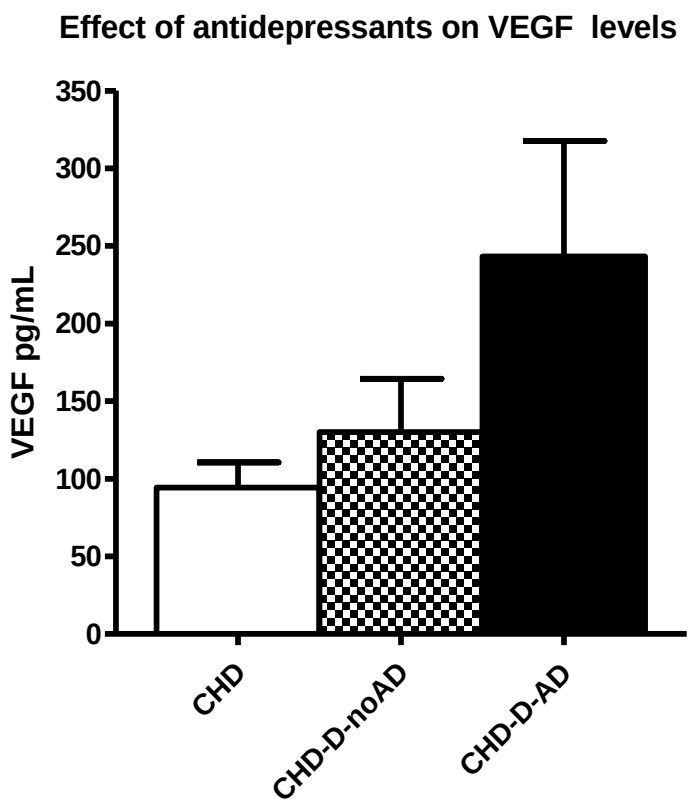


Figure 3.35 The effect of antidepressants on VEGF levels (pg/mL) in plasma. Data expressed as mean ± SEM, (CHD, n=29; CHD-D-noAD, n=13; CHD-D-AD, n=8).

Antidepressants treatment appeared to have an effect in elevated levels of VEGF in CHD depressed patients who were on antidepressants medications (CHD-D-AD, n=8) when compared with antidepressant-free CHD depressed (CHD-D-noAD, n=13) and CHD non-depressed individuals (CHD, n=29). The result was not statistically significant but showed a strong trend (p=0.05).

Table 3.6 Analysis of covariates age, gender, and antidepressant usage in relation to the outcome of the biological factors assessed in CHD patients with and without depression

	gender	age	antidepressants
CRP	F(1,51)=0.01, p=0.92	F(1,51)=1.30, p=0.17	F(1,51)=0.73, p=0.40
GR IC50-DEX	F(1,25)=1.32, p=0.26	F(1,25)=0.09, p=0.77	F(1,25)=1.87, p=0.18
Plasma Cortisol	F(1,54)=1.26, p=0.27	F(1,54)=0.07, p=0.79	F(1,54)=0.16, p=0.69
Cortisol Awakening Response Increase	F(1,50)=0.03, p=0.86	F(1,50)=0.45, p=0.50	F(1,50)=0.02, p=0.90
Diurnal Salivary Cortisol	F(1,50)=0.02, p=0.90	F(1,50)=0.34, p=0.07	F(1,50)=0.37, p=0.55
TRP	F(1,42)=0.14, p=0.72	F(1,42)=3.02, p=0.09	F(1,42)=0.31, p=0.58
Heart rate	F(1,51)=0.01, p=0.92	F(1,51)=0.83, p=0.37	F(1,51)=2.83, p=0.10
KYN	F(1,42)=2.15, p=0.15	F(1,42)=0.27, p=0.61	F(1,42)=0.002, p=0.99
KYNA	F(1,42)=1.01, p=0.32	F(1,42)=1.52, p=0.23	F(1,42)=0.62, p=0.44
3-HK	F(1,42)=2.11, p=0.15	F(1,42)=0.05, p=0.82	F(1,42)=1.80, p=0.19
3-HAA	F(1,42)=0.08, p=0.78	F(1,42)=0.44, p=0.51	F(1,42)=2.53, p=0.12

The table above demonstrates the analysis (GLM) of the effect of age, gender, and antidepressants treatment on serum CRP levels, plasma cortisol levels, peripheral GR resistance, cortisol awakening response, diurnal salivary cortisol levels, heart rate, and circulatory tryptophan, kynurenine, kynurenic acid, 3-HK, and 3-HAA metabolites. There were no significant effects of the three covariates on the biological factors investigated ($p>0.05$).

Chapter 4 DISCUSSION

4.1 Summary of the Findings

The present results show that in CHD patients, depression is accompanied by elevated levels of inflammation in the context of low cortisol output, GR resistance, and increased activation of the kynurenine pathway of tryptophan metabolism.

Although, CHD patients without depression also exhibit high inflammation as measured by circulatory levels of CRP in the serum, the level of inflammation is even higher in cases of comorbidity with depression (Figure 3.1). Consistent with high CRP in CHD depressed, candidate gene expression analysis also reveals significantly increased in IL-6 gene expression (Figure 3.3), the pro-inflammatory cytokine which stimulates the release of CRP. Both peripheral CRP (Figure 3.2) and IL-6 gene expression (Figure 3.5) are associated with the severity of depression.

Association between CHD and depression in relation to inflammation has been reported extensively, and as a focus of this PhD project, the possible underlying mechanisms leading to the higher inflammation in CHD patients with depression were investigated. In fact, the present study extends the findings by assessing in various aspects the glucocorticoid signalling as a potential contributor factor in this specific population. The results reveal that the depressed group exhibit insufficient glucocorticoid signalling possibly due to the decreased hormone bioavailability as well as attenuated glucocorticoid responsiveness. Indeed, CHD depressed patients display significantly lower levels of cortisol both in plasma (Figure 3.12) and saliva (Figure 3.14). In addition, the negative correlation between cortisol awakening response and CRP demonstrates that depression is associated with inflammation in the presence of blunted HPA axis responsiveness (Figure 3.15). In addition to a reduced availability of the hormone, CHD patients with depression represent diminished responsiveness to the effect of cortisol not only as a result of a

decrease in the number of GR as represented by significantly lower expression levels of GR mRNA (Figure 3.17), but also due to alteration in GR sensitivity as shown by less functional ability of the receptor to respond to glucocorticoids (Figure 3.26). GR resistance is also found to be associated with the severity of depression (Figure 3.27).

The result of the *in vitro* effect of medications on the GR functional properties reveals that antidepressants clomipramine (Figure 3.28) and citalopram (Figure 3.29) increase the GR sensitivity in depressed CHD patients. However, omega-3 fatty acids increase the GR sensitivity in both CHD patients with and without depression (Figure 3.30).

The present results also confirm that elevated inflammation is associated with effects on the serotonergic system through activation of the kynurenine pathway of tryptophan metabolism. The data reveal that CHD patients with depression exhibit lower levels of tryptophan together with higher IDO activity, as measured by KYN/TRP ratio (Table 3.5), which in turn is associated with an increased 3-HK level (Figure 3.31). In addition, elevated inflammation is found to be associated with increased activity of IDO enzyme (Figure 3.32) and high levels of neurotoxic 3-HK (Figure 3.33). Therefore, the results suggest that activated inflammatory response may lead to increased rate of tryptophan degradation and diversion of the kynurenine pathway towards the neurotoxic branch in CHD patients with depression.

Finally, for all of these biological factors assessed in this study, antidepressants were investigated as cofactors (Table 3.6) to understand whether or not antidepressant treatment had any effect to the outcome, and based on the results no effect was found on any of these biological factors. However, it is important to note that only 39.3% of the depressed CHD patients were under antidepressants treatment (Table 3.4), that brings into

attention the importance of screening, monitoring, and managing depression in this group of patients. In fact, more than half of the CHD patients with depression are not taking any antidepressants which not only would improve their depressive status, but would also have beneficial impact on prognosis of their cardiac condition.

4.2 Elevation of Inflammation in CHD Patients with Depression:

Inflammation has been assessed as a first approach in this PhD thesis hypothesising that CHD depressed individuals have higher circulating levels of CRP as compared to CHD patients without depression.

As mentioned before, CRP is clinically used as a biomarker of inflammation and indeed is the best biological marker for detection of inflammatory response (Willerson and Ridker, 2004). In addition, CRP is known as a mediator and marker of atherosclerosis and has been consistently reported to be a potent predictor of future vascular serious events including myocardial infarction, ischemic stroke, and sudden cardiac death. This predictive value has been shown to be independent of sex, age, blood pressure, cholesterol, smoking, and diabetes (Ridker, 2003, Ridker, 2007). Furthermore, elevation of inflammation and increased CRP concentration has been implicated in pathogenesis of depression and has been described in more details in section 1.2.5.

The present result indicates that indeed CHD patients with depression show considerably higher levels of CRP in serum as compared with non-depressed indicating elevation of inflammation in this group. As illustrated in Figure 3.1, the level of circulatory CRP is more than 3 mg/L in CHD patients without depression which is considered as a high risk factor for cardiovascular events as defined by the American Heart Association (see section 3.2.1), and even significantly higher in CHD depressed more than 5 mg/L exhibiting the higher levels of inflammation. This is a significant cause of concern since even a small elevation in CRP levels greatly increases the risk of heart attack, angina, and other cardiovascular complications.

In fact, the high CRP levels in CHD patients without depression itself represent the inflammatory activation in these patients. The endothelial damage of the cerebral vasculature as a result of high inflammation has been postulated as one of the major contributing factor for development of depressive disorder (Halaris, 2013). The cytokine and inflammatory hypothesis of depression has been described before in support to the critical role of inflammation in development and prognosis of depression (sections 1.2.1 &1.2.5). Recently, attention has been paid to the BBB hypothesis as one of the potential mechanisms underlying pathogenesis of psychiatric conditions including depression in relation to inflammation. The hypothesis suggests breakdown of BBB and its abnormal communication leading to breaching the gate and permitting penetration of inflammatory molecules into the brain (Shalev et al., 2009). CRP has been shown to be associated with increased BBB permeability (Hsuchou et al., 2012). The possible routes by which inflammatory cytokines are able to passively penetrate into the CNS have been described before in section 1.2.5. In accordance with findings of this PhD thesis, a very recent systematic review and meta-analysis of longitudinal studies concluded a significant association between elevation of the two inflammatory markers CRP and IL-6 with subsequent development of depressive symptoms (Valkanova et al., 2013). Indeed, the present results also suggest an association of both CRP and IL-6 with the severity of depressive symptoms in this study population of CHD patients (Figure 3.2 & Figure 3.5, respectively).

Significantly increased in IL-6 gene expression in CHD patients with depression in this study, as demonstrated in Figure 3.3, is consistent with higher CRP levels in this group. As mentioned before, IL-6 is the most potent inflammatory marker that induces the release of CRP and other pro-inflammatory cytokines. Pro-inflammatory cytokines are directly involved in prognosis of atherosclerosis and pathogenesis of CHD. In fact, the

release of these cytokines as a consequence of endothelial damage leads to thrombogenesis (Koenig, 2001, Mulvihill and Foley, 2002). IL-6 is believed to be a contributing factor in the stimulation of the processes leading to the formation and propagation of coronary plaque including release of adhesion molecules from endothelium and platelet aggregation (Barton, 1996). Indeed, studies have shown the expression of IL-6 gene transcripts and presence of IL-6 protein in human atherosclerotic lesions (Rus et al., 1996, Seino et al., 1994). In addition, expression and plasma concentration of IL-6 and CRP as its by product have been reported to be indicator of the intensity of inflammatory induced coronary plaque and are associated with instability of the plaque and vulnerability to rupture (Ikeda et al., 2001).

Therefore, it appears that elevated inflammation in depressed CHD patients can potentially contribute to worsening their cardiac condition. Indeed, the directionality of the depression-inflammation has been evaluated prospectively in relation to elevation of both CRP and IL-6 and their association with depressive symptoms, and the study suggests augmented inflammation preceded by depression as one of the mechanisms through which depression adversely affects cardiac health (Stewart et al., 2009).

Overexpression of IL-6 is also shown to be associated with aging. Age-associated elevation in IL-6 has been argued to account for clinically important late-life phenotypic changes and frailty as well as development of chronic inflammatory conditions in relation to advanced age (Ershler and Keller, 2000). The association between cardiovascular disease and inflammation and in particular increased in IL-6 levels as a key mediator of CRP production is the cause of concern due to increased risk of myocardial infarction and severe cardiac complications in this population. In addition, inappropriate elevations in IL-6 and CRP among the elderly have been linked to onset of disabilities including

muscle atrophy, arthritis, and osteoporosis (Ferrucci et al., 1999). Furthermore, joint elevation in the levels of IL-6 and CRP has been shown to be associated with remarkably increased mortality in a population of old people (Harris et al., 1999). These findings are important to be discussed considering the age of the present participants (Table 3.2) indicating the greater risk of disability, cardiovascular events, and mortality in this CHD depressed group who are showing elevated levels of both IL-6 expression and CRP concentrations.

Having discussed the findings in regards to IL-6 gene expression, the other results of testing the second hypothesis of this PhD, that CHD depressed have greater gene expression of cytokines than non-depressed, show that gene expression of IL-1β (Figure 3.6), TNF-α (Figure 3.8), and NF-kB (Figure 3.10) did not differ in CHD patients with and without depression. Despite the studies reporting increased level of these inflammatory biomarkers in MDD, the finding has not been confirmed by all studies (Haack et al., 1999, Kagaya et al., 2001). In addition, the protein levels of these cytokines have not been measured in this study, and since CHD is also associated with elevation of inflammatory cytokines, the circulatory levels might be already elevated in both groups with no difference between the two. It is also relevant to note that although mediated by other pro-inflammatory cytokines as well, IL-6 preserves a primary role in regulation of acute phase responses (Maes et al., 2011, Sherwood et al., 2007).

Taken altogether, CHD patients present on-going and chronic inflammation which potentially contributes to development of depression with the consequence of further activation in inflammatory processes which in turn can lead to further endothelial damage and serious cardiac complications. These findings confirm inflammation as a common link between heart disease and depression.

4.3 Insufficient Glucocorticoid Signalling in CHD Patients with Depression

4.3.1 HPA Axis Disturbance and Reduced Glucocorticoid Bioavailability

Assessing the HPA axis activity as a main regulatory system in the control of stress response and inflammation revealed very interesting findings. In this PhD, it was hypothesised that CHD patients with depression would show HPA axis abnormalities by demonstrating higher levels of cortisol both in plasma and saliva. The idea of the hypothesis came from the evidence in regards to the hyperactivity of the HPA axis, which is observed in patients with MDD in the presence of GR resistance (section 1.2.4).

Surprisingly, the present data show that compared to CHD non-depressed, depressed patients have actually lower levels of cortisol as elucidated by a single measurement in plasma (Figure 3.12) as well as repeated measures in saliva (Figure 3.14). HPA axis responsiveness was addressed by assessing its activation followed by waking up and measuring the cortisol response within the first hour of awakening that is considered as a reliable biological marker for the HPA axis activity (Pruessner et al., 1997). Indeed, a very high correlation between plasma and salivary cortisol has been reported in more recent studies investigating HPA axis function and recommending salivary cortisol measurements as reliable marker of HPA axis assessment (Petrowski et al., 2013). The result reveals significantly lower total cortisol output during the first hour post awakening as well as blunted response to the stress of awakening in CHD depressed patients (Figure 3.14). In fact, the cortisol awakening response is found to be significantly lower in CHD depressed individuals with no peak observed at 30 min which is expected in a healthy

response. Indeed, depressed patients have lower delta cortisol levels at 30 minutes post awakening compared with non-depressed group (section 3.3.2).

Despite the popularity of studies showing higher levels of cortisol in MDD as a result of hyperactivity of the HPA axis and presence of GR resistance, not all the studies have found the same results. Indeed, decreased morning cortisol in both serum and saliva samples were reported in depressed as well as vulnerable individuals in the community (Strickland et al., 2002). While some studies report findings in regards to hyperactivity of the HPA axis and elevation in cortisol levels observed in old depressed patients (Gotthardt et al., 1995, O'Brien et al., 1996), other studies explore the opposite findings suggesting hypoactivity of the HPA axis and hypocortisolism associated with late-life depression (Morrison et al., 2000, Oldehinkel et al., 2001). Indeed, a more recent study suggested the association of late-life depressive symptoms with both hyper and hypoactivity of the HPA axis and proposing the possible distinct mechanisms involved (Penninx et al., 2007). As mentioned in section 1.2.4, decreased levels of cortisol have been consistently shown in stress related conditions including atypical depression, PTSD, chronic fatigue syndrome and fibromyalgia. Some studies suggest physical frailty and exhaustion as underlying mechanism associated with HPA axis insufficiency among elderly with depression (Fries et al., 2005, Morrison et al., 2001, Oldehinkel et al., 2001).

Inconsistent findings have also been reported in regards to the association of cortisol with depression in patients with cardiovascular diseases. In the Heart and Soul Study, Otte and colleagues have found an association between depression and elevated urinary cortisol in CHD patients. However, increased cortisol has not been found to be related to the worsening of cardiac function in these patients (Otte et al., 2004). In another study in CHD patients, the results have shown an association of depression with flatter diurnal

cortisol rhythms which in turn contributed to prognosis of coronary atherosclerosis (Bhattacharyya et al., 2008). Lack of association between depressive symptoms and diurnal cortisol profile has been also reported in patients following hospitalization with an acute coronary syndrome (Molloy et al., 2008).

In general, chronic inflammatory exposure in CHD is one of the stressful challenges that these patients face, and adaptation to such condition require involvement of neural-endocrine-immune mechanisms (Chrousos and Gold, 1992, McEwen, 1998). The impact of exposure to chronic or extreme stress on the neurobiology of the HPA axis is well documented. Indeed, prolonged activation of the HPA axis has an adverse effect on health outcomes both physically and emotionally (Sapolsky et al., 2000). Some studies have shown an association of hypocortisolemia with physical complains and chronic fatigue after long term HPA axis overstimulation under chronic physical stress (Fries et al., 2005, Hellhammer et al., 2004). The activity of the HPA axis in the concept of cortisol awakening response provides the individual with energetic boost needed for the diurnal activity, and it has been suggested that lower awakening cortisol can predict greater degree of physical symptoms and fatigue during the day (Adam et al., 2006).

Therefore, considering all the findings described above, the present lower cortisol levels in the CHD depressed patients could be explained by exhaustion of the HPA axis which no longer respond sufficiently to the stress with secretion of the endocrine glucocorticoid. In fact, it can be postulated that these patients had already been under a prolonged period of HPA axis hyperactivity as a result of their chronic inflammatory condition and now the endocrine system is exhausted to produce more cortisol.

There are also other possibilities that can be suggested in relation to the HPA axis hypoactivity observed in CHD patients in the present study. The study by Pickering and colleagues proposed an association between obesity and MDD episodes with atypical features (Pickering et al., 2007). In addition, another study by Bryan and colleagues found that MDD patients with diabetes experience more physical symptoms of MDD with more atypical features (Bryan et al., 2008). Considering the well-known association of obesity and diabetes with cardiovascular disease, it can be expected that atypical subtype of depression could be strongly associated with heart disease. On the other hand, patients with atypical depression have been suggested to display a hypoactive state of the HPA axis mainly associated with decline in upstream secretagogues and specially CRH deficiency (Gold and Chrousos, 2002). The present demographic characteristic of the patients in relation to their cardiac conditions (Table 3.3) show that patients have BMI around 30 kg/m^2 being borderline overweight and obese according to National Heart, Lung, and Blood Institute definition (BMI kg/m^2: 18.5-24.9 healthy weight, 25-29.9 overweight, 30-39.9 obese, >40 extreme obese). Therefore, it would be possible that CHD depressed patients in this study are displaying atypical features of depression and thus hypoactivity of the HPA axis. However, the information in regards to the classification of depression is not available in this PhD to reach this conclusion.

The investigation in regards to the relationship between HPA activity and elevated inflammation in this study population also reveals that cortisol awakening response is negatively associated with CRP, so the lower cortisol awakening response is followed by higher inflammation as indicated by the levels of CRP (Figure 3.15). Therefore, this finding elucidates that in CHD patients, depression is associated with inflammation in the presence of blunted cortisol response to the awakening and in the other words attenuated HPA axis reactivity.

Taken altogether, it can be postulated that endogenous cortisol secretion due to hypoactivity of the HPA axis is insufficient to limit inflammation in CHD patients with depression leading to higher inflammatory response in these population.

4.3.2 Attenuated Glucocorticoid Responsiveness

Assessing GR was one of the main interests of this study due to the crucial role of this receptor in the negative feedback regulation of the HPA axis and also its immunosuppressive and anti-inflammatory effect through transrepression activity. Considering the evidence in regards to the impact of prolonged inflammation in GR alteration (section 1.2.5) and also observations of GR resistance in patients with MDD (section 1.2.4), this PhD hypothesised that CHD patient with depression exhibit GR resistance displaying lower number and sensitivity of GR. The present data indeed demonstrated that compared to CHD patients without depression, depressed CHD patients show attenuated glucocorticoid responsiveness as assessed by both expression and function of GR.

4.3.2.1 Decreased GR Number

Peripheral GR levels have been investigated through measuring total GR gene expression. Although animal studies have shown a similar pattern of upregulation and downregulation of GR both in the immune system and the CNS (Spencer et al., 1991), the findings in clinical studies in regards to decreased in GR levels (either measuring directly GR number or assessing GR gene expression) in depressed patients rely on evaluation of the peripheral levels due to limited access to the brain tissue (Pariante and Miller, 2001).

Based on the result in regards to GR gene expression presented in Figure 3.17, CHD patients with depression showed lower levels of total GR mRNA expression than CHD patients without depression indicating a decrease in the levels of these receptors in depressed group. It can be therefore suggested that these patients would have less GR available for responding to the glucocorticoids.

4.3.2.2 Alteration in GR Sensitivity

In vitro evaluation of GR function in PBMC revealed that GR sensitivity was reduced in CHD depressed patients who show less inhibition of LPS stimulated IL-6 in response to GR mediated dexamethasone effect (Figure 3.25). Decreased ability of GR indicates higher level of glucocorticoids is required to obtain 50% inhibitory effect in CHD patients with depression compared to non-depressed individuals (Figure 3.26).

The present reduction in GR sensitivity together with a decrease in gene expression of the receptor found in CHD depressed patients is consistent with the observation of higher inflammation in this group. Since glucocorticoids exert anti-inflammatory effects and inhibit the expression and production of IL-6 in the body, an impairment in GC signalling in terms of availability and function on depressed CHD patients leads to elevation of IL-6 levels, and thus CRP as its hepatic by-product. Indeed, an attenuated inhibitory response of the immune cells to the well-established immunosuppressive effect of GCs has been shown by *in vitro* studies in patients with MDD; this is while healthy subjects normally exhibit marked inhibition of cytokine production and suppression of immune activation *in vitro* followed by exposure to dexamethasone (Pariante and Miller, 2001).

As earlier described, the present CHD non-depressed patients have shown high levels of inflammation as measured by CRP levels. It is suggested that long-term inflammation can lead to GR alteration as mentioned before. Therefore, it can suggested that on-going and chronic inflammation in CHD patients could affect GR function, and the consequence of inflammatory induced GR impairment would lead to even higher inflammation that was shown in depressed CHD patients. These findings can further confirm the evidence for bidirectional association between inflammation and glucocorticoid receptors.

In addition, the findings of this study also reveal the association between GR resistance and the severity of depressive symptomatology (Figure 3.27) that is in line also with the association of inflammatory markers IL-6 and CRP with the severity of depression as discussed in section 4.2. These results suggest that more severe depression is associated with less GR functioning in combination with higher inflammation in this CHD population.

Although the present results could explain the elevation of inflammation in relation to diminished glucocorticoid responsiveness in CHD depressed patients, how is it possible that these patients are showing hypoactivity of the HPA axis in the presence of GR resistance, whereas previous published studies on MDD patients have reported hyperactivity of the HPA axis in the presence of GR resistance due to alteration in negative feedback inhibition of cortisol secretion mediated by GR? To address this question it would be first needed to explain the methodological approaches in regards to GR assessment.

As the result of this PhD showed, CHD depressed patients exhibited decreased sensitivity of GR to dexamethasone as measured *in vitro* directly on PBMC, and so the GR

sensitivity was assessed at the peripheral level. However, another standard method which is designed to evaluate GR function in relation to the HPA axis activity and assessing the integrity of GR-mediated negative feedback is the *in vivo* dexamethasone suppression test (DST) in which HPA axis response is measured following dexamethasone administration. Although the test has been used extensively for verification of HPA axis dysregulation, due to its modest sensitivity (40-50%) and lack of specificity to depression, it has been refined by combining the DST and CRH challenge. In this test, which is known as DEX/CRH test, following pre-treatment by dexamethasone, ACTH response to CRH is suppressed. Thus, GR-mediated feedback inhibition can be challenged via DST and DEX/CRH tests at the level of pituitary, and are used to quantify indirectly HPA axis dysregulation (De Kloet et al., 1998).

Performing the *in vivo* tests described above, would provide further support for confirming the GR resistance found in this population of study. However, since the *in vivo* assessment has not been carried out in this project, there is no evidence whether alteration in GR sensitivity may directly affect the HPA axis that might be intact otherwise. It could be possible that GR present in HPA tissue display different mode of action from the peripheral once found in PBMC. Some studies reveal different GR functioning in different tissue. For instance, animal studies have shown the alteration in both expression and function of GR in the pituitary contributing to HPA axis hyperactivity and still functional GR in the hippocampus (Schmidt et al., 2009).

It should be also considered that impairment within the HPA axis that can affect the production and/or release of cortisol contributes to hypoactivity of the HPA axis and insufficient levels of glucocorticoids. While not performed in this study, measurements of ACTH and CRH as higher-level regulatory factors controlling cortisol release could

provide some insight into the possible mechanisms underlying the dysregulation of the HPA axis. Indeed, attenuated synthesis and release of upstream signalling molecules and their blunted response potentially lead to insufficient GC signalling implicated in neuropsychiatric conditions including chronic fatigue syndrome (Scott et al., 1998), fibromyalgia (Torpy et al., 2000), and atypical depression (Geracioti et al., 1997). In addition, understanding HPA axis activity requires consideration of confounding factors which can potentially affect the neuroendocrine function and cortisol levels (Cleare, 2003). Although not assessed in this PhD, measurements of activity levels and sleep disturbance implicated in hypocortisolaemic conditions (Cleare, 2003) could help with better interpretation of HPA axis alteration in this study population.

Taken altogether, the evidence from the investigation of the HPA axis and GR in this PhD thesis suggests that depression is associated with insufficient glucocorticoid signalling in CHD patients. The inadequate signalling capacity of glucocorticoids appears to be due to reduced cortisol bioavailability as well as lack of sufficient GR-mediated glucocorticoid response due to reduced numbers and sensitivity of the receptor. Blunted cortisol response and HPA reactivity as well as GR resistance at peripheral level are in turn associated with high inflammation in these patients.

4.4 Diversion of the Kynurenine Pathway towards the Neurotoxic Branch in CHD Patients with Depression

Having discussed insufficient glucocorticoid responsiveness which partly explained the elevated inflammation in CHD patients with depression, kynurenine pathway of tryptophan metabolism as a psychoneuroimmunologic bridge known to be implicated in inflammatory induced MDD, appeared to be an interesting and important system to investigate in this CHD population. Therefore, this thesis hypothesized that CHD patients with depression would demonstrate disturbances in tryptophan metabolism pathway through showing activation of IDO enzyme and production of neurotoxic metabolites of the kynurenine pathway.

As described in section 1.2.7, following an increased inflammatory response and IDO activation, the peripheral availability of tryptophan is reduced that in turn putatively leads to reduced serotonin synthesis in the brain. A shift towards the kynurenine pathway and the production of neurotoxic metabolites are considered to be essential steps in the pathophysiological processes. In fact, it is believed that the imbalance between the neuroprotective and neurotoxic metabolites of the system potentially contributes to depression with 3-hydroxykynurenine and quinolinic acid overcoming the kynurenic acid production.

The findings in regards to investigation of kynurenine pathway in this study are presented in Table 3.5. The results show that CHD patients with depression exhibited lower peripheral levels of tryptophan as compared to CHD non-depressed. The depressed group also display an increased in IDO activation as reflected by the KYN/TRP ratio which is in turn associated with higher levels of the neurotoxic metabolite 3-hydrokynurenine (Figure

3.31). However, such an association was not found between KYN/TRP ratio and the levels of neuroprotective metabolite kynurenic acid. The results therefore indicate that the kynurenine pathway is more likely going through the neurotoxic branch in CHD depressed persons.

As mentioned before, activated inflammatory responses have been suggested to induce depressive symptoms not only by directly affecting the brain but also modulating the serotonergic system suggesting a causal relationship between altered inflammation and the disturbances in the serotonergic system (Dantzer et al., 2011, Wichers and Maes, 2004). Inflammatory induced IDO overstimulation has been shown to be associated with depressive symptoms as a result of peripheral tryptophan depletion which in turn results in serotonin deficiency (Wichers and Maes, 2002). The animal study by Zalcman showed that following intraperitoneal injection of IL-6, neurotoxic metabolites, as measured by levels of 5-HIAA, were elevated in the prefrontal cortex and hippocampus (Zalcman et al., 1994).

In regards to cardiovascular diseases, previous studies have shown decreased tryptophan concentration and higher KYN/TRP ratio coincided with increased neopterin concentration (marker of monocyte/macrophage activation) in CHD patients compared to healthy control (Wirleitner et al., 2003b). In a line with the findings of this PhD thesis a very recently published study also revealed an association between KYN/TRP ratio and CRP as well as neopterin in CHD patients suggesting IDO activity to be related with immune and inflammatory activation (OZKAN et al., 2014). IDO activity is believed to be associated with risk factors for atherosclerosis and prognosis of cardiovascular disorders (Niinisalo et al., 2008, Pedersen et al., 2011, Pertovaara et al., 2007). One study

also suggested an association between depressive symptoms and carotid atherosclerosis in relation to the effect of IDO activation (Elovainio et al., 2011).

Although kynurenine pathway has been studied in relation to inflammation induced depression, and also IDO activity in relation to inflammation and atherosclerosis, to my knowledge, no studies to date have been investigated IDO activity and the kynurenine pathway in CHD patients in relation to inflammation and with comorbid depression. This study indeed has taken into consideration the levels of CRP and IL-6 confirming inflammatory activation in the present population.

Considering CHD as an inflammatory condition, inflammation appears to be associated with the activation of the kynurenine pathway in CHD patients with and without depression as indicated by the correlation between KYN/TRY ratio and CRP in this study population (Figure 3.32). The result is in line with the findings in relation to the association between inflammation and IDO activity. In addition, the correlation between CRP and 3-HK (Figure 3.33) might indicate the effect of inflammation in triggering kynurenine pathway activation and production of neurotoxic metabolites.

Taking into account the previous results in relation to insufficient glucocorticoid signalling and consequently elevated inflammation in CHD patients with depression, it appears that high inflammation in these patients activates the kynurenine pathway of tryptophan metabolism via increased IDO activity. An increased rate of tryptophan degradation results in measurable decline in tryptophan levels in CHD depressed patients. The consequence of the increased inflammatory response in turn may lead to a diversion of the kynurenine pathway towards the neurotoxic branch in CHD patients with depression. The schematic diagram of these findings is illustrated in Figure 4.1.

Measuring quinolinic acid level in plasma in patients would confirm further the diversion of kynurenine pathway towards the neurotoxic branch. In addition, the kynurenine metabolites were measured in the periphery, and it would have been ideal to measure the associated metabolites in the cerebrospinal fluid (CSF) as well. However, this could not be possible since quinolinic acid is notoriously very difficult to measure, and the CSF samples of the patients were not available.

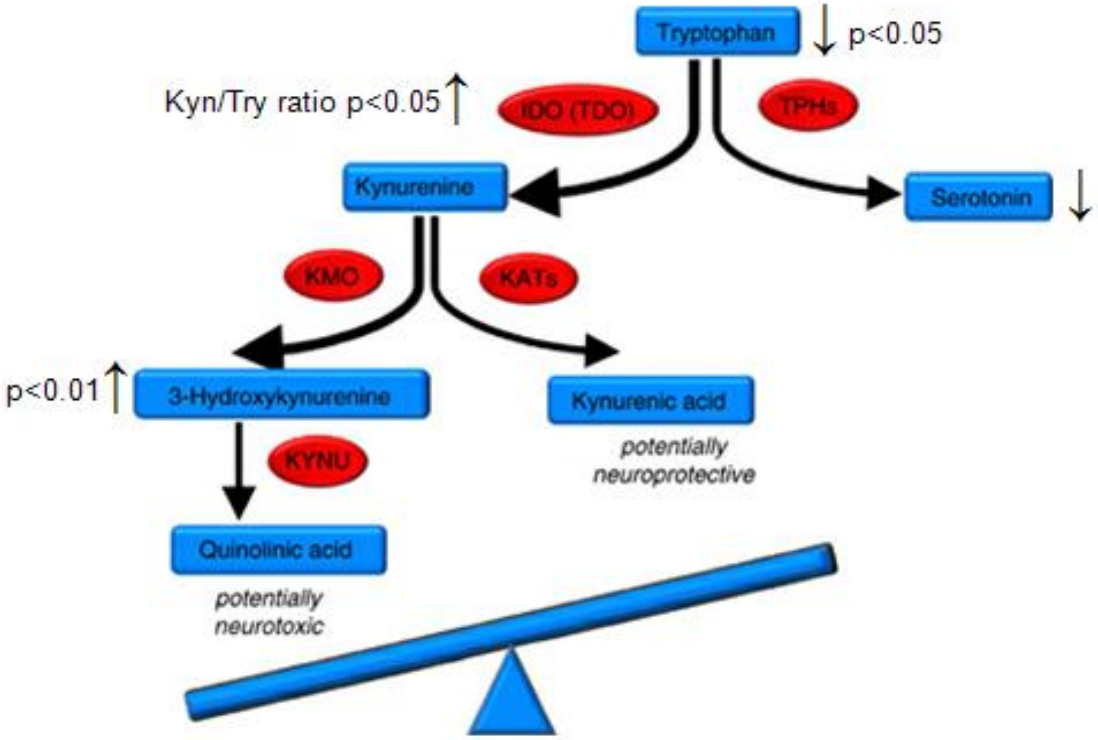


Figure 4.1 Diversion of the kynurenine pathway towards the neurotoxic branch in CHD patients with depression based on (Zunszain et al., 2012).

4.5 Increased VEGF in CHD Patients with Depression

Overexpression of VEGF is implicated in the pathophysiology of diseases such as malignancies and promotes vascular permeability in involved tissues (Grothey and Galanis, 2009, Thurston, 2002). In addition, VEGF circulatory levels are associated with disease progression in autoimmune conditions (Carvalho et al., 2007). VEGF has been studied extensively in relation to tumour angiogenesis and has been indeed suggested as a target for anticancer therapy (Albini and Sporn, 2007, Carmeliet, 2005).

VEGF has been also studied in CHD patients. Some studies have shown increased VEGF levels in patients with coronary atherosclerosis (Blann et al., 2002) and thus indeed suggesting an association between prognosis of coronary atherosclerosis and elevated VEGF (Fleisch et al., 1999), and also beneficial effect of statin therapy (atorvastatin) in lowering VEGF circulatory levels (Alber et al., 2002). However, no correlation between VEGF plasma concentration and severity of coronary artery disease has been also reported (Alber et al., 2005).

Since VEGF is widely expressed throughout the body, attention has been recently paid to the role and implications of this vascular permeability factor beyond the vasculature and specifically in the brain. Known as a neurotrophic factor, VEGF is thought to exert neuroprotection (Storkebaum et al., 2004) and neurogenesis (Jin et al., 2002) effect in addition to vasculogenesis. The direct neurotrophic activity of VEGF in the brain has been shown to be exerted through stimulating axonal outgrowth and enhancing cell survival (Sondell et al., 1999), protecting hippocampal neurons against glutamate mediated toxicity (Matsuzaki et al., 2001), and influencing synaptic plasticity (Licht et al., 2011) and transmission (McCloskey et al., 2005).

Since VEGF plays a critical role in both the brain and vascular system, and considering the relationship between CHD and depression, VEGF has been suggested to be one of the molecular links underlying the comorbidity of these two disorders (Warner-Schmidt and Duman, 2008). Therefore, this PhD thesis hypothesised that CHD depressed patients would display higher plasma levels of VEGF as compared to CHD individuals without depression.

There are considerable discrepancies among studies investigating VEGF in depression. While some studies have reported increased VEGF mRNA (Iga et al., 2007), serum (Kahl et al., 2009) or plasma (Lee and Kim, 2012, Takebayashi et al., 2010) levels in patients with MDD, others have found significant decreases in VEGF peripheral levels (Dome et al., 2009, Isung et al., 2012, Katsuura et al., 2011) associated with depression. In contrast to all these studies, VEGF protein levels have also been reported to show no difference between patients with MDD and healthy control (Kotan et al., 2012, Ventriglia et al., 2009). A very recent review assessing clinical studies on VEGF and depression speculated that elevation in VEGF levels in patients with MDD appears to be due to the response to the perceived stress associated with depression resulting in an attempted neuroprotective effect. Whereas, decreased levels of VEGF seem to be observed in treatment-resistant depressed patients whose brains are less able to undergo neurogenesis processes (Clark-Raymond and Halaris, 2013).

The present data suggested significant elevation in circulatory VEGF in CHD patients with comorbid depression (Figure 3.34). However, having investigated the effect of antidepressants, it appeared that the increase in VEGF levels in CHD depressed patients is mainly affected by antidepressant treatment (Figure 3.35). VEGF has been studied in relation to antidepressants. An increased VEGF expression in the hippocampus has been

achieved in response to different classes of antidepressants including SSRIs. In addition, VEGF signalling via its corresponding receptor (tyrosine kinase fetal liver kinase; Flk-1 or VEGFR2) has been suggested as an essential inducer of the antidepressants-mediated neurogenesis activity (Warner-Schmidt and Duman, 2007). This neurotrophic factor has been proposed to be a putative biomarker for treatment plan in depression and predictor of treatment response (Clark-Raymond and Halaris, 2013), therefore, a potential candidate for therapeutic interventions (Warner-Schmidt and Duman, 2008).

It should be noted that in the present study, VEGF was measured in the plasma. Interestingly, decreased in VEGF levels in some brain regions including hippocampus and frontal cortex, but no change in serum levels have been observed in a genetic rat model of depression (Elfving et al., 2010). Whether or not VEGF peripheral levels correlate with those in the human brain remains a future hypothesis to be investigated in clinical studies.

4.6 Increased Heart Rate in CHD Patients with Depression

The metabolic demand of the body is controlled by heart activity through its rate. The association between an increase in the basal heart rate and the progression of atherosclerosis and sudden CHD death is documented. Indeed, the heart rate is known as an independent prognostic risk factor for morbidity and mortality related to cardiovascular disease (Dyer et al., 1980). Clinical studies have shown prognostic benefit of a reduction in the heart rate following beta-blockers treatment and improvement in cardiac outcome and decreased in mortality (Ferrari et al., 2003). Elevation in the heart rate has been observed in patients with comorbid CHD and depression (Carney et al., 1988, Carney et al., 1993).

In the present study, CHD patients with depression also show a higher heart rate than those non-depressed (Table 3.3). The finding is found to be independent of beta-blocker therapy considering the fact that more than half of the patients in each group were on this medication (Table 3.4). Therefore, higher heart rate in CHD depressed patients may be an indication of adverse consequence of comorbidity of depression in this population leaving the patients at higher risk of severe cardiac events and death.

Alteration in heart rate in major depression is thought to be as a result of alteration in neural control of cardiac function. Epinephrine and norepinephrine effects on cardiac β-adrenergic receptors or an increased sensitivity of the receptor have been suggested as the underlying mechanisms. Indeed, elevation in the levels of catecholamine in CSF as well as plasma has been observed in patients with MDD (Gold et al., 2005, Grippo and Johnson, 2009). The present results showed an association between the CRP levels and heart rate (section 3.2.1) indicating that high degree inflammation could raise the heart rate in CHD patients.

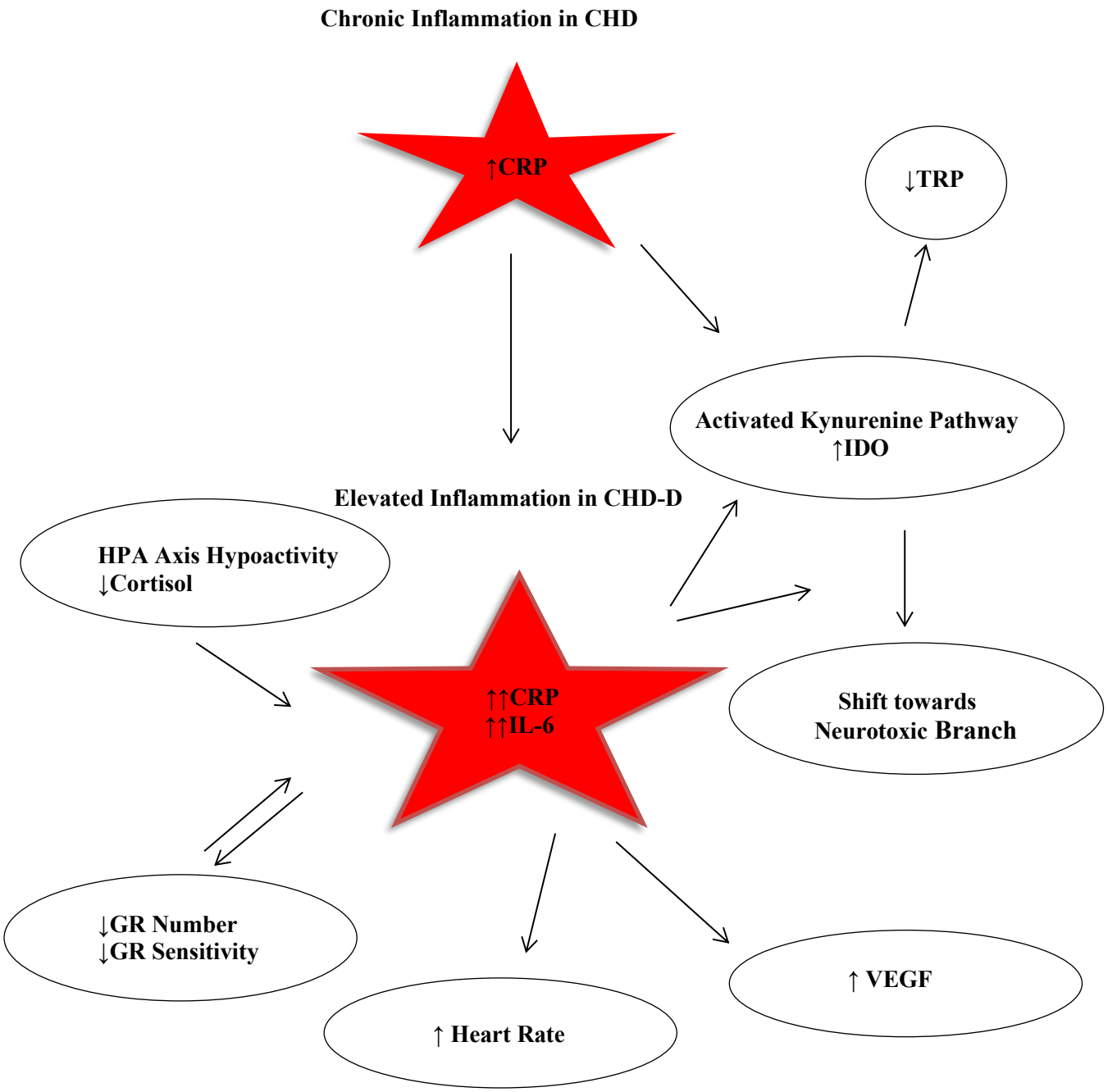


Figure 4.2 Simplified diagram representing the results of this PhD thesis

4.7 Inflammation as a Target in Treatment of Depression in CHD Patients: Evidence from the *In Vitro* Study

Having investigated inflammation in CHD patients with and without depression in this PhD thesis, and considering inflammation as a potential mechanism contributing to the development of depression in patients with CHD, and also the negative impact of comorbid depression on the cardiac outcome in CHD depression, it appears that targeting inflammation could be considered as one of the potential therapeutic strategies in increasing the treatment rates for the depression in CHD patients.

Statins are the most commonly used medications in heart disease due to their lipid lowering properties, and they are also known to exert anti-inflammatory effect (Schonbeck and Libby, 2004). There is evidence of pleiotropic functions of statins in diminishing levels of the inflammatory marker CRP that appears to be independent of its lipid lowering effect (Musial et al., 2001, Ridker et al., 1999, Ridker et al., 2001). In this study, although, more than 80% of the patients have been on statin treatment (Table 3.4), the results showed that CRP levels were higher in both CHD depressed and non-depressed and even significantly higher in the depressed group. This reveals that statin therapy alone might not have sufficient anti-inflammatory effect in CHD patients with comorbid depression. While once it was thought that a statin could be beneficial in the treatment of depression due to its effect in reducing inflammation, studies have emerged proposing the contribution of this medication to the development of depression through the lowering of cholesterol levels, which are essential for the serotonin system (Pasco et al., 2010, You et al., 2013).

On the other hand, considering the relation of inflammation in the pathophysiology of depression, treatment with anti-inflammatory agents such as cyclooxygenase-2 inhibitor has been shown to be beneficial in patients with MDD (Muller et al., 2006). In addition, as mentioned before in section 1.2.8, studies on therapeutic strategies using antidepressants report that some antidepressants appear to exhibit beneficial effect through reducing levels of inflammatory biomarkers and modulating inflammation-induced depression (Tousoulis et al., 2009) as well as attenuating neuro-inflammatory activation in depression (Lu et al., 2010, O'Sullivan et al., 2009). However, the mechanisms of actions of antidepressants as anti-inflammatory agents are still under investigations.

For the treatment strategies in reducing inflammation in CHD patients with depression, the goal would be targeting the specific mechanisms underlying the inflammatory activation in these patients. Due to major role of adequate glucocorticoid signalling in the regulation of inflammation and metabolism, studies on potential therapeutic intervention in CHD, have paid attention to the involvement of GR in the pathophysiology of atherosclerosis.

GR genes have been studied in relation to heart disease. Studies suggest variation between individuals in relation to glucocorticoid functionality. Four common glucocorticoid receptor gene polymorphisms; BclI C/G, GR-9β A/G, N363S, and ER22/23EK, have been associated with glucocorticoid sensitivity (Huizenga et al., 1998, Van Rossum and Lamberts, 2004). GR gene haplotype 3 (GR-9β A/G) related to reduced GR sensitivity and more pro-inflammatory response has been shown to be associated with risk of cardiovascular disease (van den Akker et al., 2008). In addition, the investigation in regards to the association of these genes with depression in a population of CHD

patients has revealed that this common genetic variant of GR is also associated with depression and has been suggested as a vulnerability factor for depression in CHD (Otte et al., 2009). Moreover, in CHD patients the therapeutic effect of beta-blockers and calcium blocker has been investigated on GR suggesting the mechanisms for their anti-inflammatory activity through increasing GR levels (Ji et al., 2010). Therefore, GR appears to be a strong link between CHD and MDD, and a potential candidate to be targeted in treatment of depression in heart disease.

As the result of this study showed and discussed in detailed (section 4.3.2), CHD depressed patients exhibited lower sensitivity of GR (Figure 3.26) as one of the potential mechanisms leading to elevated inflammatory response (Figure 3.3 & Figure 3.1) in these patients. Evaluating GR function, It was also hypothesised that antidepressant and omega-3 treatment *in vitro* would improve GR-mediated glucocorticoid response in CHD depressed patients who showed decreased GR sensitivity. Indeed, testing the hypothesis in this population revealed that the GR functional responsiveness appeared to be improved by the *in vitro* effect of antidepressants clomipramine (Figure 3.28) and citalopram (Figure 3.29) as well as EPA (Figure 3.30); that potentially may lead to more effective response to the anti-inflammatory and immunosuppressive activity of glucocorticoids.

The effects of antidepressants in regulation of GR function have been suggested by various studies (Carvalho and Pariante, 2008, Pariante and Miller, 2001). Tricyclic antidepressant desipramine has been shown to enhance GR function by facilitating GR translocation from cytoplasm into the nucleus *in vitro* (Pariante et al., 1997). Clomipramine has been observed to reduce GR function *in vitro* in healthy individuals, and the absence of the effect was reported in treatment resistance depressed patients

(Carvalho et al., 2008). Although clomipramine was found to increase GR sensitivity in CHD depressed patients in the present study, it would not be considered clinically as the treatment choice in pharmacotherapy of depression in this population due to cardiotoxic effects of tricyclic antidepressants and the risk of overdose death liability associated with this class of antidepressants (Alvarez and Pickworth, 2003, Roose and Glassman, 1994). However, it can be argued that the finding might be relevant in general in comorbidity of depression in medically-ill patients experiencing chronic inflammatory conditions.

The SSRI antidepressants and specially citalopram and sertraline are recognised to be safe and effective, thus the first-line antidepressants for CHD patients who suffer from moderate to severe as well as recurrent depression (Glassman et al., 1993, Glassman et al., 2002, Lespérance et al., 2007). Studies on the effects of citalopram in relation to heart disease and depression, have been shown the beneficial actions of this antidepressant in patients with comorbid cardiovascular disease and depression through improvement in endothelial function (van Zyl et al., 2009). Based on the result of *in vitro* assessment of citalopram on GR function (Figure 3.29), the present study also reveals that this SSRI antidepressant may also improves GR sensitivity in CHD depressed patients who exhibit GR resistance. Although, this finding does not explain the exact mechanism through which citalopram increases GR responsiveness, it might indicate the potential mechanism of anti-inflammatory action of this antidepressant that seems to be through modulation of GR function.

The present data in regards to the effect of EPA on GR functionality *in vitro* show an increase in the sensitivity of GR in both CHD patients without depression and those with depression when EPA was co-incubated with dexamethasone. This finding may also reveal the potential mechanism of anti-inflammatory effects of omega-3 PUFAs through

increasing GR function thus a better response to the cortisol. Indeed, clinical studies have shown the inverse association of omega-3 and the two inflammatory biomarkers CRP and IL-6 in a population of CHD patients (Farzaneh-Far et al., 2009). The authors have suggested omega-3 fatty acids may prevent adverse cardiac events possibly through mechanisms that lead to the inhibition of inflammation. Omega-3 PUFAs are found to influence capacity of monocytes for the synthesis of cytokines such as IL-1 and TNF. Indeed, they are found to promote the cytokine suppression at the levels of transcription and regulation of their gene expression (Simopoulos, 2002). Omega-3 PUFAs synthesise classes of lipid mediators such as resolvins, lipoxins, maresins, and protectins that are known to resolve inflammation (Lee, 2012). Decreased leucocyte chemotaxis, inhibiting adhesion molecule expression, and diminishing interaction between leukocyte and endothelium are other anti-inflammatory activities of these fatty acids (Calder, 2013).

Based on the result of this PhD thesis, it has been shown that CHD patients with and without depression exhibited high levels of inflammation, and that CHD depressed individuals showed even higher inflammation and in the presence of GR resistance when compared with CHD non-depressed. In addition, it was observed that there was an association between CRP, IL-6, and GR resistance with the severity of depressive symptoms. Furthermore, the *in vitro* findings revealed that EPA might lower the inflammatory status through increasing GR sensitivity in both CHD patients with and without depression. Therefore, it can be postulated that omega-3 would be potentially an effective therapeutic medication in CHD patients with or without comorbid depression.

The significant of this finding relies on the fact that omega-3 can be favourable choice of medication due to their lack of side effects. Indeed, this agent is known as a safe therapeutic component with potentially no known side effect for CHD patients. In

addition, omega-3 is believed to be both protective and therapeutic agent for depression (Su et al., 2000). Furthermore, the effect of omega-3 has been already investigated in clinical trials and has been suggested to be an effective mediation in reducing depressive symptoms in MDD patients (Nemets et al., 2002) as well as TRD individuals (Puri et al., 2002). Moreover, epidemiological studies reveal the protective effect of dietary omega-3 on adverse cardiac events (Breslow, 2006, Harris et al., 2007, Lee et al., 2008).

Although not measured in this study, low levels of omega-3 PUFAs have been reported in patients with MDD. The brain contains high concentration of PUFAs which are essential for the proper functioning of the CNS (Bourre et al., 1991). Since they are the major component of neuronal cell membranes, their alterations cause disturbances in membrane microstructure leading to alteration in signal transduction and increasing the risk of depression (Horrobin and Bennett, 1999, Logan, 2003).

Deficiency of omega-3 fatty acids has been considered as a crucial factor contributing to the pathophysiology of heart disease and depression and is a potential link between the two (Severus et al., 1999). Omega-3 deficiency in platelet membrane is associated with a decreased heart rate variability which is the risk for sudden cardiac death (Christensen et al., 1997) and has been reported to be remarkably lower in depressed patients with CHD (Carney et al., 1995).

It must be noted that the effects of the antidepressants clomipramine and citalopram, and EPA on GR function were assessed *in vitro*, thus with no involvement of pharmacodynamic and pharmacokinetic parameters which influence *in vivo* studies. This study assessed the *in vitro* effect of EPA on improving GR sensitivity in CHD patients with or without depression that in turn could result in reducing inflammation in these

patients. However, the effect is suggested to be investigated further through *in vivo* observations.

CHD patients remain under the conditions of chronic inflammation, and the present results showed the deleterious effect of comorbidity with depression on GR and HPA axis leading to a further augmentation of inflammation. Therefore, managing inflammation-induced depression and preventing further elevation of inflammation appear to be the vital approach to treatment of these patients. This would be only possible by targeting the specific mechanisms underlying inflammatory activation in these patients one of which could be GR alteration. It can be postulated that co-administration of suitable anti-inflammatory medications with effective antidepressants might have strongest effect and more rapid response in CHD patients suffering from depression.

4.8 Strengths, Limitations, and Future Work of the Study

This project is the first to investigate detailed mechanisms associated with inflammatory activation in CHD patients with and without depression by assessing inflammatory response, HPA axis activity, GR sensitivity and kynurenine pathway in the same population. Indeed, compared to other studies, this study is the only one looking at the potential cause and consequences of inflammation associated with depression in CHD at the same time. Although other published studies have assessed HPA axis activity in CHD patients with depression, this study has revealed the HPA axis disturbances together with blunted cortisol awakening response in relation to elevated CRP levels and in the presence of GR resistance at the periphery. In addition, kynurenine and tryptophan levels have been previously assessed by other studies and more recently in relation to elevated inflammation, however, the kynurenine pathway has never been investigated before in this specific population.

It should be noted that investigating glucocorticoid signalling through measurements of GR expression and function which directly assesses the responsiveness status is more accurate and stable indicator than only measuring cortisol levels due to such a circadian fluctuation during a single day. However, the present study assessed both direct and indirect functional measurements of GR by investigating GR expression and function, as well as circulating cortisol levels both with a single measure in plasma and repeated measures in saliva.

This study also benefits from the fact that patients did not differ in terms of using concomitant medication (besides antidepressant) which can be a complication in such

complex conditions. Also, gender did not have an effect in all the findings that is also a common complication in stress related conditions.

This PhD study mainly concentrated on the role of inflammation involved in the pathophysiology of depression investigated in a specific population of CHD patients. However, major scientific theories proposed in relation to the potential causes and possible mechanisms involved in the development of depression have been mentioned in section 1.2.1 and cannot be ignored. Rather conflicting results yielded by different published studies in relation to depression appear to be mainly due to the heterogeneity of this disease and specifically when it is comorbid with other physical illnesses; and it seems to be even more diverse in elderly population.

The cross-sectional nature of this PhD thesis offered some valuable findings for better understanding of the role of inflammation in CHD patients with and without depression by investigating the mechanisms underlying the pathophysiology of inflammation-induced depression and the biologically related consequences of comorbidity of depression in CHD patients. However, further prospective investigation would enable examining the longitudinal association between heart disease and depression in relation to inflammation.

All the patients have been followed up by UP-BEAT study for four years every six months recording the information in regards to their depression and cardiac status, and these data could be used for future studies in order to investigate the development of depression and cardiovascular related complications including MI and death. In addition, DNA samples were collected from 400 CHD patients with and without depression that will be genotyped for detecting polymorphisms associated with inflammation, GR, and serotonin. The genetic approaches would advance our understanding of the underlying

common mechanisms involved in the co-morbidity of these two devastating disorders. The ultimate goal will be identifying the genetic and/or biological markers that can be used for prediction or early detection of depression in CHD patients.

Detecting inflammatory-induced depression will have important translational significance as it will identify biomarkers that predict future development of depression and thus identify subjects that may benefit from targeted therapeutic prophylactic intervention. Moreover, it will confirm inflammation as a pharmacological target to develop new antidepressants, and will detect biomarkers that could be used to monitor changes in vulnerability.

One of the limitations of this study is the sample size of the group with depression, and specifically the very small sample size when the groups were divided for the antidepressants usage; this may have contributed to the non-significant findings in these analyses. In addition, in some experiments only a subtype of the patients was analysed due to the lack of availability of the samples. For example, for saliva samples there were cases that the amount of samples collected were not sufficient to measure; in addition, not all the patients who provided blood samples also provided saliva sample. This also happened in the GR function assessment that PBMC were not available for all the patients that were investigated for their gene expression levels. Furthermore, it should be noted that the study participants were elderly and that the old age is also associated with inflammation, higher prevalence of depression, and increased risk of cardiovascular complications. However, the findings of this study were controlled for age. Moreover, assessments of confounding variables such as activity levels and sleep disturbance that can potentially affect HPA axis function have not been measured by this study.

CONCLUSION

This PhD thesis provides valuable finding in regards to the well-established association between CHD and depression with an insight into the role of inflammation. Based on the results of this study, it can be concluded that chronic inflammation in patients with CHD potentially contributes to the development of depression which in turn leads to further activation of inflammatory processes as reflected in joint elevation in IL-6 expression and CRP levels both with a significant association with the severity of depressive symptomatology in this population. CHD depressed patients display hypoactivity of the HPA axis possibly due to the exhaustion of the system because of long-term inflammation. The elevated inflammation in CHD patients with depression is concomitant with insufficient glucocorticoid signalling as a result of decreased hormone bioavailability, as observed through lower levels of plasma and salivary cortisol as well as attenuated glucocorticoid responsiveness due to the decreased expression and function of GR. Inadequate glucocorticoid signalling associated with inflammation may make CHD patients with depression less equipped to respond to stress. Blunted cortisol response and HPA reactivity as well as GR resistance at peripheral level are indeed associated with high inflammation in these patients showing dysfunction of the regulatory mechanisms to limit the inflammation. The consequence of increased inflammation associated with depression adversely affects the patients with CHD. Indeed, CHD individuals with depression show the involvement of the serotonergic system through the activation of the kynurenine pathway of tryptophan metabolism as evidenced by an increase in IDO activity. An increased rate of tryptophan degradation results in measurable decline in tryptophan levels in CHD depressed patients. The consequence of the increased inflammatory response in turn may lead to diversion of the kynurenine pathway towards the neurotoxic branch in CHD patients with depression. In addition, having higher heart

rate in CHD patients with comorbid depression may leave the patients at higher risk of severe cardiac events and death.

These findings may provide a better understanding of the role of inflammation in the pathogenesis of depression in coronary heart disease and an ample support of the observations in regards to inflammation as a remarkable link between these two devastating disorders. Therefore, enhancing the knowledge of the relationship between heart disease and depression, and uncovering in details the specific mechanisms involved, would open the possibility for promising therapeutic strategies. The strategies that would target directly those affected mechanisms, and avoid trying unnecessary conventional therapies. Indeed, the management of inflammation-associated depression through anti-inflammatory approaches remains as a novel strategy for the treatment of depression in patients with CHD and other chronic inflammatory disorders. Therefore, this study provides evidence in regards to improvement of GR function *in vitro* in response to the effect of antidepressants and anti-inflammatory agents which may lead to a more effective response to the anti-inflammatory and immunosuppressive activity of glucocorticoids. Specifically, omega-3 PUFAs would potentially increase GR sensitivity in both CHD patients with and without depression. Upon validation of their remarkable effect on GR function by *in vivo* studies, this study recommends that omega-3 may be potentially an effective therapeutic medication in CHD patients with or without comorbid depression.

Recognition and management of depression in CHD patients remain essential to avoid its deleterious effect associated with the negative cardiac prognostic impact. The ultimate goal is to attenuate the adverse cardiac outcome in depressed heart disease patients, to improve the quality of life, and also to decrease the mortality rate among these patients.

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APPENDIX

Appendix-A Patient Information and Invitation letter

Patient Information Sheet

REC REF 09/H1103/19

Inflame-BEAT: Understanding the role of inflammation in predicting depression in patients with heart disease

Reference number: Participant number:

Part 1

We would like to invite you to participate in this research study. Before you decide it is important that you understand why the research is being done and what it will involve. Please take time to read through this information carefully and discuss it with others if you wish. Please ask us if there is anything that is not clear or if you would like more information. Thank you for reading this.

What is the purpose of the study?

Depression and heart disease are common health problems. Patients who suffer from heart problems may have an increased risk of becoming depressed. Unfortunately, depression in patients with heart disease increases the risk of having future cardiac problems and death. It is unclear why depression affects people with heart disease.

In our research study we are interested in identifying what the main risk factors that predispose to depression are. In particular, we want to know whether there are any genes or molecules that increase the risk of developing depression, either alone or by interacting with environmental factors such as stress, and infections. Part of the reason why some people become ill may lay in genetic differences between people, in the same way that we are different in the colour of our eyes, hair etc. These genetic differences also correspond to differences in certain molecules in our blood and saliva. To achieve this, we will compare the blood, saliva and genetic make-up of people with heart disease with and without current diagnosis of depression with the make-up of people with similar characteristics but no history of heart disease or mental health problems.

By understanding the role of molecules in the blood that make some people more susceptible than others to becoming ill; and by understanding how environmental factors work together with molecules and genes in causing depression in the future will allow us to predict and treat better these devastating disorders.

In conclusion, the type of genetic analysis that we carry out is only for research purposes and does not at present produce clinically relevant results.

Why have I been invited?

We are interested in patients who have been diagnosed with heart disease. Your GP thinks you have (or have had) heart disease and would like you to discuss participating in the study with a

member of the study team. We want about 800 with heart disease to help us with the study. However, no information will be passed to the study team without your consent.

Do I have to take part?

No. It is entirely up to you whether or not you decide to participate in this study and a member of the research team will discuss this with you. They will describe the study and go through this information sheet, which we will then give to you. If you decide you would like to take part you will be asked to sign a consent form but will still be free to withdraw at any time without giving a reason. If you decide not to take part, or withdraw at any time, this will not affect the care you receive from your GP or any other health professional involved in your care.

What will happen to me if I take part?

We would like to follow people for 1 year. Initially a researcher will contact you to arrange a convenient time to meet, either at your home, at King's College London or at your GP's surgery. At this meeting the researcher will tell you more about the research and answer any questions you may have. The researcher will then contact you again 2 days later to discuss whether you wish to participate in the study. They will ask you to sign a consent form and then complete a questionnaire. The questionnaire will ask you questions about symptoms of heart disease and depression, your treatment, and your social circumstances. In this occasion we will ask from you a small sample of blood and a cheek swab sample. The cheek swab is a simple procedure (we can show you the kit and illustrate the technique) that collects some of the cells from the inside of your cheek. This part will take about an hour. After this we will contact you every 6 months, during one year and arrange a convenient time either personally or by phone and ask you to complete a shorter questionnaire again asking questions about your symptoms and mood. This will take about half an hour. In total you will meet with the researcher 3 times. All information you give us will be treated in the strictest confidence. You will be given a study number so that no information you give us has your name on it. We will not interfere with your normal treatment in any way.

A medically trained researcher will take the blood sample using disposable sterile equipment. It will only take few minutes as for any routine blood sample. If you are unable or unwilling to give a blood sample it is also possible to perform genetic analysis from cheek swab samples, a simple procedure that (we can show you the kit and illustrate the procedure) collects dead cells present in your saliva and in your mouth. From the cheek swab sample we cannot measure level of medication or look at expression of genes, we can only extract a small amount of DNA. Therefore we prefer to ask for a blood sample to guarantee a better quality of our results and make the most out of your generous help.

A researcher will demonstrate how to collect saliva sample and will provide you with the tubes required. The level of some proteins contained in the saliva can give us an indication of differences in the level of stress experienced by healthy volunteers and people suffering from mental illnesses.

We will also ask you for some of your time to collect clinical and social demographic information using standardised research instruments: diagnostic interview, symptoms rating scale, socio demographic interview and neuropsychological tests. Depression and life events questionnaires can be potentially embarrassing or upsetting, as could disclosure of symptoms during face to face interview. If you are not interested in giving the blood, you can still take part to the rest of the study.

What is being tested?

We may use your blood and the cheek swab sample to: 1) Measure the level of hormones and proteins contained in the blood serum and in the blood cells; 2) Look at the variation in some genes of interest in the cheek cells.
A medically trained researcher will take the blood sample and conduct the cheek swab, using disposable sterile equipment. It will only take few minutes.

You will continue to receive your care as usual through your GP or other health professionals. We will not interfere with any care you receive.

What will I have to do?

We would ask you to meet with the researcher at 6 monthly intervals at a time and place convenient to you. The researcher will contact you to arrange these meetings. At the meetings we would also ask you to answer the questions asked by the researcher.

What will happen to my sample and medical information?

Your DNA sample and medical information will be assigned a number and will be bar coded with this. This number will be how researchers keep track of samples and information. Of the collected blood, serum and lymphocyte/monocyte cell fractions (PBMC) will be prepared, thereafter stored at -180°C, until molecular and functional tests will take place. Genomic DNA will be collected at certain points in the study from the PBMC, but strictly used for polymorphism studies only and as indicated. After completion of the studies left over genomic DNA will be destroyed. All information will be anonymised.

Expenses and payments

You will be reimbursed any travel costs associated with participating in this study.
As a gesture of gratitude for your participation you will be reimbursed for your time.

Will personal information about me be kept confidential?

Your confidentiality is of the up most importance to us, therefore we have put together a number of steps to safeguard your information. We will follow ethical and legal practice and all information about you will be handled in confidence. Your research record will be kept in a separate secure file at the Institute of Psychiatry (IoP) and your medical records will not hold any of your results from this research. Your blood or cheek swab cannot be linked back to you as your sample will be coded with your participant number (not your name). Under no circumstances will any of your personal details be passed to anyone outside the IoP. Your participation is strictly confidential. Your medical information and any results will be put on a computer and stored (in coded form) in an electronic database. It is likely that the results of this study will be published. However your name will not appear on any publications or reports about this research.

What are the possible risks of taking part?

The risks involved are those of ordinary blood tests such as small pain and occasionally a small bruise around the area from where the sample has been taken. There is no risk involved in the collection of cheek swab. Since your care is not being affected in any way, there are no other risks to participating in the study.

What are the possible benefits of taking part in the study?

Although we cannot promise that this study will be of immediate benefit to you, by taking part you will help to increase the knowledge of what causes people to be more susceptible to depression and heart disease.
We hope that in the future this knowledge may lead to the development of better treatments and improve prevention of these illnesses. You may benefit from this.

Will I be contacted again?

If you agree, we may contact you in the future to invite you to participate in other studies.
If you would prefer not to be contacted this will not affect your participation in this study.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

Part 2

What will happen if I don't want to carry on with the study?

You are free to withdraw from the study at any time without giving reason, and your care will not be affected. If you withdraw from the study information already collected may still be used.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to Dr Livia Carvalho who will do his best to answer your questions (Contact number: 0207 848 0352). If you remain unhappy and wish to complain formally, you can do this through the Institute of Psychiatry Complaints Procedure. Details can be obtained from the Institute of Psychiatry.

Confidentiality

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the practice will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the study?

The results should be published in a medical journal within 18 months of the end of the study. They will also be fed back to you doctor, and to appropriate non-statutory agencies. We will send you a newsletter reporting the findings of this study. You will not be identified in any report or publication arising from the study.

Who is organising and funding the study?

The study is funded by the Department of Health and is sponsored by King's College, London. Your doctor is not being paid anything extra for including you in the study.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given a favourable opinion by the Research Ethics Committee.

You will be given a copy of this Patient Information Sheet and a signed consent form to keep.

Where can I get further information about the study?

If you would like further information please contact:

Dr Livia Carvalho

Stress, Psychiatry and Immunology Laboratory
Institute of Psychiatry, King's College London
125 Coldharbour Lane, SE5 9NU
Tel. +4402078480352
Fax: +4402078480986
Email: l.carvalho@iop.kcl.ac.uk

Appendix-B Appointment Confirmation

Institute of Psychiatry

at The Maudsley

Stress, Psychiatry and Immunology

Dr. Livia A Carvalho

Department of Psychological Medicine
Centre for the Cellular Basis of Behaviour, The James Black Centre
Institute of Psychiatry, Kings College London
125 Coldharbour Lane
London SE5 9NU

Tel + 44 (0) 207 848 0352
Fax + 44 (0) 207 848 0986
l.carvalho@kcl.ac.uk

KING'S College LONDON *Founded* 1829

University of London

Appointment Confirmation

London, DD/MM/YY

Dear

Thank you for agreeing to participate in our research project.
I confirm our appointment on the ..... at ..... AM.

On the study day, you will be able to find me at:

Address

16 De Crespigny Park, London, SE5 8AF, UK

Institute-of-psychiatry

I will be waiting for you at the main reception.

You will be reimbursed for your travel expenses (provided you leave the receipt with us).

In the unlikely event that you are not able to come on the agreed day, please contact me asap on 0772 500 0710.

Thank you very much in advance,

Yours sincerely,

Dr. Livia Carvalho
CCBB, The James Black Centre
Institute of Psychiatry, King's College London
125 Coldharbour Lane
London, SE5 9NU
Tel. +4402078480352
Email: l.carvalho@iop.kcl.ac.uk

Appendix-C Participant Consent Form

Participant Consent Form REC REF 09/H1103/19

A copy of this consent form (signed and dated) will be given to you.

Inflame-BEAT: Understanding the role of inflammation in predicting depression in patients with heart disease

Reference number: Participant number:

My signature below indicates that:

- **I have read this form and the research has been explained to me.**
- **I have been able to discuss the research and ask questions. I am satisfied with the answers I have received.**
- **I have been given the time to consider whether or not to take part in this study.**
- **My participation in this research is completely voluntary.**
- **It has been explained to me that I am free to withdraw from the study at any time without giving reason, and without my medical care or legal rights being affected.**
- **I am willing for researchers to seek information from doctors that have treated me for mood disorder and to review my medical records.**

The following consent elements are optional. Please initial each box to indicate your consent:

▪ **I agree to provide blood sample**	**YES**	**NO**
▪ **I agree to provide a sample of my DNA using a cheek swab.**	**YES**	**NO**
▪ **I am willing to be contacted in the future regarding participation in other studies.**	**YES**	**NO**

Participant's Name: _____
(*Please print*)

Participant's Signature: _____
Date:____/_____/_____

(Day/Month/Year)

Individual obtaining the Participant's Consent: I verify that I have explained the nature of this study to the participant and to the best of my knowledge addressed any questions the participant has asked.

Name: _____
(Please Print)
Signature: _____ **Date:**____/_____/_____
(Day/Month/Year)
Position: _____

Appendix-D Participant Questionnaire

Questionnaire

Name: _____Date:_____

Address: _____

Telephone number: _____Email:_____

Age _____ _____ Date of Birth_____

Weight_____ Height_____

1) Do you take any medication on a daily basis?

_____

2) Did you take anything different yesterday? Today?

_____

3) Do you have any pets at home?

_____

4) Are you feeling good? Any flu or cold signs?

_____

5) Did you have breakfast today? At what time?

---

6) Are you currently working? If YES, what is your job title?

_____

7) Would you like to be called for related studies?

_____

Appendix-E Beck Depression Inventory

Name_____ Date_____

BDI

On this questionnaire are groups of statements. Please read each group of statements carefully. Then pick out the one statement in each group that best describes the way you have been feeling the PAST WEEK, INCLUDING TODAY. Circle the number beside the statement you picked. If several statements in the group seem to apply equally well, circle each one. Be sure to read all the statements in each group before making your choice.

1 0 I do not feel sad
1 I feel sad
2 I am sad all the time and I can't snap out of it
3 I am so sad or unhappy I can't stand it

2 0 I am not particularly discouraged about the future
1 I feel discouraged about the future
2 I have nothing to look forward to
3 I feel that the future is hopeless and that things cannot improve

3 0 I do not feel like a failure
1 I feel I have failed more than the average person
2 As I look back on my life, all I can see is a lot of failures
3 I feel I am a complete failure as a person

4 0 I get as much satisfaction out of things as I used to
1 I don't enjoy things the way I used to
2 I don't get real satisfaction out of things anymore
3 I am dissatisfied or bored with everything

5 0 I don't feel particularly guilty
2 I feel guilty a good part of the time
3 I feel quite guilty most of the time
4 I feel guilty about everything

6 0 I don't feel I am being punished
1 I feel I may be punished
2 I expect to be punished
3 I feel I am being punished

7 0 I don't feel disappointed in myself
1 I am disappointed in myself
2 I am disgusted with myself
3 I hate myself

8 0 I don't feel I am any worse than anybody else
1 I am critical of myself for my weaknesses and my mistakes
2 I blame myself all the time for my faults
3 I blame myself for everything bad that happens

9 0 I don't have any thoughts of killing myself
1 I have thoughts of killing myself, but I would not carry them out
2 I would like to kill myself
3 I would kill myself if I had the chance

10 0 I don't cry anymore than usual
1 I cry more now than I used to
2 I cry all the time now
3 I used to be able to cry, but now I can't cry even though I want to

11 0 I am no more irritated now than I ever am
1 I get annoyed or irritated more easily than I used to
2 I feel irritated all the time now
3 I don't get irritated at all by the things that used to irritate me

12 0 I have not lost interest in other people
1 I am less interested in other people than I used to be
2 I have lost most of my interest in other people
3 I have lost all of my interest in other people

13 0 I make decisions about as well as I ever could
1 I put off making decisions more than I used to
2 I have greater difficulty in making decisions than before
3 I can't make decisions at all anymore

14 0 I don't feel I look any worse than I used to
1 I am worried that I am looking old or unattractive
2 I feel there are permanent changes in my appearance that make me look unattractive
3 I believe that I look ugly

15 0 I can work about as well as before
1 It takes extra effort to get started at doing something
2 I have to push myself hard to do anything
3 I can't do any work at all

16 0 I can sleep as well as usual
1 I don't sleep as well as I used to
2 I wake up 1-2 hours earlier than usual and find it hard to get back to sleep
3 I wake up several hours earlier than I used to and cannot get back to sleep

17 0 I don't get more tired than usual
1 I get tired more easily than I used to
2 I get tired from doing almost anything
3 I am too tired to do anything

18 0 My appetite is no worse than usual
1 My appetite is not as good as it used to be
2 My appetitie is much worse now
3 I have no appetite at all anymore

19 0 I haven't lost much weight, if any, lately
1 I have lost more than 5 pounds; I am purposely trying to lose weight
2 I have lost more than 10 pounds in weight by eating less
3 I have lost more than 15 pounds: Y__ N__

20 0 I am no more worried about my health than usual
1 I am worried about physical problems such as aches and pains, upset stomach, or constipation
2 I am very worried about physical problems and it is hard to think of much else
3 I am so worried about my physical problems that I cannot think about anything else

21 0 I have not noticed any recent changes in my interest in sex
1 I am less interested in sex than I used to be
2 I am much less interested in sex now
3 I have lost interest in sex completely

Appendix-F Saliva Sample Participants Letter

University of London

Dr Livia Carvalho

CCBB, The James Black Centre
Institute of Psychiatry,
Kings College London
125 Coldharbour Lane
London SE5 9NU

Tel + 44 (0) 207 848 0352
Fax + 44 (0) 207 848 0986
L.carvalho@iop.kcl.ac.uk

Date

Dear

Thank you for agreeing to participate in our research. Depression and heart disease are common health problems, with heart problems increasing the risk of depression whilst depression may increase future cardiac risk. In our research we are interested in identifying early biological markers that predispose people to depression. To measure levels of stress, and the response to stress, we analyse hormones present in saliva. This valuable data can be used in conjunction with previously gathered data to search for effects on heart disease progression and treatment.

Please find enclosed six cotton swabs, with instructions for use. There is one swab for each sampling time:

1. At time of waking (tube marked 0)
2. 15 minutes after waking (tube marked 15)
3. 30 minutes after waking (tube marked 30)
4. 60 minutes after waking (tube marked 60)
5. At 2pm (tube marked 2)
6. At bedtime (tube marked B)

To prevent any void samples, please could you leave the cotton swab in place for **two minutes.**

I would be grateful if you could also fill in the enclosed questionnaires at the time of sampling, to be returned with the completed samples, within 2 weeks of this letter.

Many thanks for your continuing support that makes this valuable research possible.

Yours sincerely,

Dr. Livia A Carvalho, INFLAME-BEAT Program Leader

Enc. *Cortisol instructions*
Sampling questionnaire
Beck questionnaire
Return stamped addressed envelope

Any questions please call on 07824 701249 – thank you. (Jennie Parker).

Appendix-G How to Collect the Saliva Samples

How to Collect the Saliva Samples

You should have six tubes, each with a different number, relating to saliva collection times. Only **one** sample is required at each of the specified times.

x 6 plus questionnaire, "measuring your biological levels of stress".

1. Take the test tube marked with the appropriate number and remove the lid:

2. Remove the inner tubing to expose the cotton roll:

3. Place the cotton roll in your mouth and **chew on it**; it is essential that you keep it there for **2 minutes** to ensure a sufficient sample is collected.

4. Place the cotton roll back into the inner tube and replace the lid/label.

5. Immediately place the sample tube in the fridge.

6. Repeat the above at 15, 30 and 60 minutes after the waking sample, and again at 2pm and bedtime.

Please remember to fill in the questionnaire at the time of each sample.

Once collected, return all samples/questionnaire in the pre-paid envelope. Thank you.

Appendix-H A Step by Step Guide for the Saliva Collection

Measuring your biological levels of Stress:

A step by step guide for the saliva collection

Date of saliva collection: _____ Name: _____

Saliva Code_____ GAP ID: _____ GAPBarcode_____

Baseline □ 3 Months Follow up □ 12 Months Follow up □

Wake up (before 10 a.m.).

Immediately after waking up collect your saliva putting the Sorbette ***under the tongue*** and leaving it for 60 seconds, then put it back in the tube **marked 0**.

Write here the **EXACT TIME OF AWAKENING: _____**

Try to sit down and relax in the next hour. **YOU CANNOT BRUSH YOUR TEETH AND CANNOT HAVE ANYTHING TO EAT OR DRINK FOR THE NEXT HOUR.** If you need, you can drink water, but only immediately AFTER you have taken the sample.

15 minutes after waking up, collect your saliva using the **tube marked 15**.

- What time is it now? _____
- What were you doing before giving the sample?_____

_____

- Did you accidentally have anything to eat or drink before taking the sample? If yes, please describe it here: _____

_____

- Did you have any difficult or tense situation, unpleasant thoughts or any kind of pain before taking this sample? If yes, please describe it here:

_____

30 minutes after waking up, collect your saliva using the **tube marked 30**.

- What time is it now? _____
- What were you doing before giving the sample? _____

_____

- Did you accidentally have anything to eat or drink before taking the sample? If yes, please describe it here: _____

_____

- Did you have any difficult or tense situation, unpleasant thought or any kind of pain before taking this sample? If yes, please describe it here:_____

_____

60 minutes (1 hour) after waking up collect your saliva using the **tube marked <u>60</u>**.

- What time is it now? _____
- What were you doing before giving the sample? _____

_____

- Did you accidentally have anything to eat or drink before taking the sample? If yes, please describe it here: _____

_____

- Did you have any difficult or tense situation, unpleasant thought or any kind of pain before taking this sample? If yes, please describe it here:_____

_____

<u>*** You can now have breakfast and brush your teeth!</u>**

<u>***</u>**

At 2pm collect your saliva using the **tube marked 2**.

<u>You should not eat or drink anything, or do not brush your teeth in the 30 minutes before noon.</u>

- What time is it now? _____
- What were you doing before giving the sample? _____

_____

- Did you accidentally have anything to eat or drink before taking the sample? If yes, please describe it here: _____

_____

- Did you have any difficult or tense situation, unpleasant thought or any kind of pain before taking this sample? If yes, please describe it here:_____

_____

At bedtime collect your saliva using the **tube marked B**.

<u>You should not eat or drink anything, or do not brush your teeth in the 30 minutes before bedtime.</u>

- What time is it now? _____
- What were you doing before giving the sample? _____

_____

- Did you accidentally have anything to eat or drink before taking the sample? If yes, please describe it here: _____

_____

- Did you have any difficult or tense situation, unpleasant thought or any kind of pain before taking this sample? If yes, please describe it here:_____

_____

Store the tubes away from the heat and direct sunlight and <u>put them into the fridge as soon as possible</u>.

Please note name and time of any **medication taken <u>today</u>** (including the contraceptive pill): _____

_____

Do you have any **medical problem**? If so, please list them here_____

_____

If you are female: Please indicate the age of your <u>first</u> menstrual cycle: _____

And please indicate the date of the <u>first day of your last</u> menstrual cycle: _____

If you have any questions about any of these instructions please call **Dr Livia Carvalho on 0207 8480352 or 07725000710; or Jennie Parker on 07824701249. Thank you.**

Appendix-I Salivary Cortisol Guideline for the Researcher

Salivary Cortisol Guideline:

Sample Collection

- SWABS should be used to collect saliva samples; that changes according to age:

    - Up to 6 months, Salimetrics Infant's Swab (SIS)
    - Between 6 months and 6 years, Salimetrics Children's Swab (SCS)

      Both of these are ordered from Salimetrics (http://www.salimetrics.com/collection-supplies)

    - For adults, we use Salivette® Cortisol

      (http://www.sarstedt.com/php/main.php?inhalt=produktfamilien.php?gruppe_id=12&SID=c6a286dfa05454251e68f3e1850ae3f1)

- The salivettes should be labelled by numbers: 1, 2, 3, 4, 5 and 6; and then below the number the indicated time: 0 min, 15 min, 30 min ,60 min, 12 noon, 8 pm, respectively.

- The researcher should encourage the participant to wake up between 5-10 am on the day they intend to collect the sample. It should be explained to the participants that the awakening sample should not be taken earlier than 5 am or later than 10 am; and if that is the case, they should leave the collection of samples for another day.

- It is highly recommended that the researcher should ask all participants to take the awakening sample as soon as they wake up followed by recording the exact time of awakening. It should be make clear to the participant that if the first sample has not been taken immediately after awakening or maximum 5 minutes after, the collection must be left for another day.

Dealing with Time Errors:

- It is highly important that the researcher explain to all the participants and make them understand about the necessity of recording the exact time for collecting each sample. However if:

    - The time record is missing for one or more points, the researcher should contact and check immediately with the participant in case they might remember the time of the sample collection (at least approximate time).

- The time sheet is missing completely and no information in regards to the time of sample collection could be obtained; the sample will be considered and included as normal using the standard time.

Dealing with Sampling Errors:

- It is important for the researcher to go through the saliva cortisol data for each participant to double check the sampling time recorded for each data point. In cases that any sample has not been collected exactly at the expected time, the data can be included if the sample has been taken within the following range :

  - For 15 min sample: ± 5 min
  - For 30 min sample: ± 10 min
  - For 60 min sample: ± 15 min
  - For 12 noon sample: ± 1 hr
  - For 8 pm sample: ± 1 hr

- For the samples that the cortisol level is above or below the detectable range, the following values should be considered:

  - 82.7 for high values
  - 0.33 for low values

- Please note that if a sample contains less than 5ul saliva it will not be measured, as results would not be reliable enough. The values will be only reported for the samples that can be used.

Dealing with Missing Data:

- If the data for the time point 0 from awakening is missing, the CAR cannot be calculated, but the diurnal cortisol can be calculated considering the data for 15 min as the first sample.

- If the data for 15 min or 30 min is missing, the average of the two points before and after the missing data should be used (For missing the data for 15 min, the average of awakening and 30 min can be used; and for 30 min, the average of 15 min and 60 min). If the data for both 15 and 30 min are missing, CAR cannot be calculated, but the sample can be included for calculating the diurnal cortisol.

- If the data for 60 min is missing, the sample is excluded for calculating the CAR but included for diurnal cortisol.

- If the data for 12 noon and/or 8 pm is missing, the sample is excluded for diurnal cortisol but included for calculating the CAR.

Appendix-J PBMC Isolation, Freezing and Storage

Peripheral Blood Mononuclear Cell (PBMC) Isolation Using Ficoll Paque, Freezing and Storage of Viable Cells

Introduction

This instruction describes peripheral blood mononuclear cell (PBMC) isolation using Ficoll Paque, freezing and storage of viable cells.

Necessary materials

15-25 ml of Heparinized blood	9 ml sodium heparin tubes; Greiner 455051
- PBS, sterile, pH 7.4	stock 10X PBS; Lonza BE17-517Q - 1:10 diluted in milli-Q water - check osmolarity (must be 260 - 320) - check pH (must be 7.3 - 7.5) - fill out and autoclave - after opening, expired after 1 month
- Ficoll-Paque PLUS	GE Healthcare 17-1440-03
- RPMI 1640, with 25 mM Hepes, with Ultraglutamin-1	Lonza BE12-115F/U1
- Fetal Bovine Serum (FCS)	Lonza DE14-801F,
- PEN-STREP (10.000 U Penicillin/ml, 10.000 U Streptomycin/ml) stock	Lonza DE17-602E
- Dimethylsulfoxid (DMSO)	Merck 8.02912.1000
- Culture fluid 1	90% RPMI 1640, with 25 mM Hepes, with Ultraglutamin-1 + 10% FCS + 1% PEN-STREP (i.e. 5 ml PEN-STREP stock solution to 500 ml of Culture fluid) - store at 4°C - expired after 1 month
- Culture fluid 2	80% Culture fluid 1 + 20% DMSO - prepare fresh, on ice - expired after 1 day!
- 50 ml polypropylene tubes	BD Biosciences (Falcon) 352070
- Cryovials, 1.2 ml, certified Rnase, Dnase, Pyrogen and DNA-free	Simport, Canada T309-1A
- Lab-durable vinyl labels, 50mm x 16mm	eg. Brady B-427
- Suitable label printer	eg. Brady BP-4000
- Cryo 1ºC Freezing Container	Nalgene 5100-0001 - filled with 250 ml propanol-2 - refresh propanol-2 after 5 times used

Safety

Personal and environmental protection

Wear gloves when working with human blood or serum. Work in a flow cabinet level II.

Dangerous materials

compound	**classification**	**R- and S-phrases**	**cas-no**	LWCA-code
Liquid nitrogen	irritative	-	7727-37-9	-
DMSO	toxic, inflammable, explosive	-	67-68-5	3
propanol-2 (isopropanol)	lightly inflammable, irritative	R11, S(2)-7-16	78-96-6	3

Operating instruction

Note: Make sure that peripheral blood, PBS, Ficoll and centrifuge are at room temperature (~18 - 22°C).

Place Culture fluid 1 and Culture fluid 2 on ice.

1. Determine the White Blood Cell count, using a cell counter.
2. Dilute sodium-heparinized blood 1:1 with PBS at room temperature.
3. Carefully layer diluted blood on top of Ficoll (2:1, eg. 30 ml of diluted blood on 15 ml of Ficoll) in 50 ml polypropylene tubes.
4. Centrifuge 15 min at 1000 g, at room temperature, without break (light off).
5. Draw off the upper (plasma) layer, leaving the PBMC layer undisturbed at the interface.
6. Using a sterile Pasteurs pipette transfer the PBMC layer to a clean 50-ml polypropylene tube.
7. Add PBS to a total volume of 50 ml.
8. Centrifuge 10 min at 800 g.
9. Remove the supernatant.
10. Place the tube, containing the cells on ice.
11. Resuspend the cells in a certain volume of cold Culture fluid 1, eg 1.0 ml.
12. Determine the PBMC cell count.
13. Determine the exact amount of cells to freeze per vial:
    Preferably store 2 vials (15 - 25 *10^6 PBMC in a total volume of 1.0 ml per vial).
14. Label the Cryovials to be used in a proper way (5 lines on a 50mm x 16mm label):

eg. **BD-UG-5023** <Disease code[1]-Short name participant[2]-patientID of the Institute>
BD 10-MAY-83 <Birth Date, dd-mmm-yy, 2nd characteristic>
PBMC
19.2*10E6 <amount of cells>
12-JUL-08 <date (dd-mmm-yy) of sampling>

Note: In case of a follow up sample: use the same patient ID, but different date of sampling!

15. Slowly, in droplets add cold Culture fluid 2 (1:1) to the cell suspension.
16. Aliquot: 1.0 ml of cell suspension per cryovial.
17. Immediately put the vials in a Cryo 1ºC Freezing Container into the -80°C freezer.
18. Store the vials after 4 - 72 hours at -196°C in the liquid Nitrogen vessel.

Remarks

- Complete the whole procedure on the same day of blood drawing.
- Polypropylene tubes are used to prevent activation of monocytes.

Appendix-K PBMC Cell Culture Protocol

University of Roehampton London

PBMC Cell Culture Protocol

1 INTRODUCTION

This instruction describes an assay to defrost PBMC frozen (see the PBMC isolation using Ficoll Plaque, freezing and storage of viable cells Protocol) analyse GR function in vitro.

2 DEFINITIONS AND ABBREVIATIONS

3 THEORY

4 SAFETY

5 NECESSARY MATERIALS

- FALCON 3077, 96 well
- PBS x1, sterile, pH=7.2
- LPS (Sigma, Ref#L2880; 10mg)
- Dexamethasone (Sigma, Ref#D1756; 500mg, MW:392.46)
- Clomipramine (Sigma, Ref#C7291-1G; 500mg, MW:351.3)
- Citalopram hydrobromide (Sigma, Ref#C7861-10MG, MW: 405.3)
- Methyl all-cis-5,8,11,14,17-eicosapentaenoate (Sigma, Ref#E2012-1MG, MW: 316.48)
- Ethanol
- Culture fluid 1 90% RPMI 1640, with 25 mM Hepes, with Ultraglutamin-1
  + 10% FCS
  + 1% PEN-STREP (i.e. 5 ml PEN-STREP stock solution to 500 ml of Culture fluid)
  - store at 4°C
  - expires after 1 month

6 *In vitro* experiment PBMC

Final concentrations:

LPS	1 ng/ml
Dexamethasone	10^{-6}M 10^{-7}M; 3x10^{-8}M; 10^{-8}M; 10^{-9}M
Clomipramine	10^{-5} M
Citalopram	10^{-5} M
Methyl all-cis-5,8,11,14,17-eicosapentaenoate	10^{-5} M

Experiment day 1

Concentration	Dilution (μL)	CF1 volume (μL)	Final total volume (μL)
LPS 20 ng/mL	20	980	1000
DEX 2 x 10^{-4}M	8	995	1000
DEX 2 x 10^{-5}M	100	900	1000
DEX 2 x 10^{-6}M	100	900	1000
DEX 6 x 10^{-7}M	300	700	1000
DEX 2 x 10^{-7}M	335	665	1000
DEX 2 x 10^{-8}M	100	900	1000
CLOMIPRAMINE	7.1	993	1000
CITALOPRAM	8	992	1000
EPA	33	467	500
ETHANOL	33	467	500

6.1–LPS (FREEZER -20)

Product: Sigma (10mg) - Ref # L2880

a) To prepare the stock solution: Take 10 mg of LPS and add 10 mL of PBS 7.2 and stock in -20^0 C Freezer (Final concentration: 1mg/mL). Stock 3 μL per eppendorff.

b) To prepare the solution for use in the culture:

b.1) Take 1 μL of the LPS stock solution (1mg/mL) and add to 1000 μL of PBS. Use the vortex in solution. (Final concentration: 1μg/mL)

b.2) Take 20 μL of the LPS working solution (1μg/mL)) and add to 980 μL of CF1. Use the vortex in solution. (Final concentration: 20ng/ml)

6.2 – DEXAMETHASONE (FRIDGE)

Product: Sigma (500 mg) – Ref # D1756-1G – MW: 392.47

a) To prepare the stock solution: Take 10 mg of dexamethasone and add 1 mL of alcohol (Final concentration: 10mg/mL or 25.48 x 10^{-3} M).

b) To prepare the solution to use in the culture:

b.1) Take 7.8 μL (8 μL) of the dexamethasone stock solution (25.5 x 10^{-3} M) and add to 992 μL (995 μL) of CF1. (Final concentration: 2 x 10^{-4} M)

b.2) Take 100 μL of the dex solution (2 x 10^{-4} M) and add to 900 μL of CF1.
(Final concentration: 2 x 10^{-5} M)

b.3) Take 100 μL of the dex solution (2 x 10^{-5} M) and add to 900 μL of CF1.
(Final concentration: 2 x 10^{-6} M).

b.4) Take 300 μL of the dex solution (2 x 10^{-6} M) and add to 700 μL of CF1.
(Final concentration: 6 x 10^{-7} M)

b.5) Take 335 μL of the dex solution (6 x 10^{-7} M) and add to 665 μL of CF1.
(Final concentration: 2 x 10^{-7} M)

b.6) Take 100 μL of the dex solution (2 x 10^{-7} M) and add to 900 μL of CF1.
(Final concentration: 2 x 10^{-8} M)

6.3 – CLOMPIPRAMINE (FRESH)

Product: Sigma (500 mg) – Ref # C7291-1G – MW: 351.3

Prepare CMI solution always fresh (0.028M or 10mg/mL) by adding 1 mL of PBS to the 10 mg CMI weighed powder, or equivalent. Use the vortex in solution.

To prepare the solution for use in the culture

Take 7.1 μL of the CMI solution (0.028M) and add to 993 μL of CF1.
(Final concentration: 2x10^{-4} M)

6.4 – CITALOPRAM (FRESH)

Product: Sigma (10 MG) – Ref # C7861-10MG – MW: 405.3

Prepare CIT solution (0.025M or 10mg/mL) by adding 1 mL of ethanol to the 10 mg CIT powder, or equivalent. Use the vortex in solution. Store in -20ºC.

To prepare the solution for use in the culture

Take 8 μL of the CMI solution (0.025M) and add to 992 μL of CF1.
(Final concentration: $2x10^{-4}$ M)

6.5 – METHYL ALL-CIS-5,8,11,14,17-EICOSAPENTAENOATE

Product: Sigma (1MG) – Ref #E2012-1MG

Prepare EPA solution (0.003M or 10mg/mL) by adding 1 mL of ethanol to the 1 mg EPA, or equivalent. Use the vortex in solution. Store in -20ºC.

To prepare the solution for use in the culture

Take 33 μL of the EPA solution (0.003M) and add to 467 μL of CF1.
(Final concentration: $2x10^{-4}$ M)

6. 6 – Ethanol solution for adding to the control culture wells

Take 33 μL of the ethanol and add to 467 μL of CF1.

7 *In vitro* Peripheral Blood Mononuclear Cells (PBMC) Experiment:

Prepare LPS, Dexamethasone, Clomipramine, Citalopram, EPA and ETOH dilutions. Add 10µl of the dilutions to the required wells.

Take one tube of frozen PBMC from liquid nitrogen/-80^0 C freezer and transfer immediately to a 37^0 C bath. Warm up 10ml CF1 in a 15 ml tube in the water bath. While the cells still have a little bit of ice inside, transfer them to the 15mL tube with 10mL of CF1 inside. Centrifuge 800g, 5 min, RT and discard the supernatant. Then, add 1mL of CF1 and proceed to cell count.

COUNTING CELLS: to prepare the hemacytometer, first clean the hemacytometer with H2O and then with 70% ethanol. Dry it off with a Kimwipe. Put 90µl PBS on a well, add 10 µl of cell suspension (using 10-100 uL pipette) and 100uL of trypan blue Mix well with pipette. Cell count: Place hemacytometer on the counter. Centre a cover glass over the hemacytometer chambers. Fill one chamber with 10 µl of the cell dilution using a 10-100µl pipette.

Count the amount of cells to be added to each well (100K). Calculate and add CF1 for the total volume of 200uL for each well. Add the amount of cells counted to all the wells.

8 INCUBATION

The plate will be incubated on a plate shaker at 37 ^{0}C with 5% CO_2 for 24 hours.

9 AFTER 24 HOURS

Centrifuge the plate 1000g, 10 min, 4 ^{0}C. Transfer the supernatant from each well to a labelled eppendorf and store in -80^0 C freezer ready for the ELISA experiment.

10 REMARKS

11 REFERENCES

12 APPENDICES

Appendix-L IL-6 ELISA Assay

IL-6 ELISA ASSAY

For Culture Supernatants

1. INTRODUCTION

This instruction describes an assay to analyse any human cytokine with ELISA for less than 2 euros/sample. This method has been used in the past for TNF-alpha, IL-1alpha, IL-1beta, ril-1RA, INF-alpha, IL-4, IL-6, IL-8, IL-10, and IL-12p40.

2 DEFINITIONS AND ABBREVIATIONS

IL-6, interleukin 6; BSA, bovine serum albumin; PBS, phosphate buffer saline

3 THEORY

4 SAFETY

5 NECESSARY MATERIALS

- Costar 3590, 96 well
- PBS pH=7.8 (PBS pH7.8, Gibco 20012019)
- NaCl (BDH Chemicals Ltd. Poole England, Prod. 10241 / 9092083C)
- Tween 20 (Fisher Scientiffic, BP-337100)
- BSA (Sigma A9647, 50G)
- Capture antibody
  Invitrogen: Ms mAb Anti-Hu IL-6
  (REF: AHCO562 / LOT 72575914A)
- Recombinant human IL-6
  R & D system: Recombinant Human IL-6
  (Catalogue Number 206-IL / LOT OJZ0410052)
- Detection antibody
  Invitrogen: Ms mAb Anti-Hu IL-6 Biotin
  (REF: AHCO469 / LOT 72682798A)
- Required dilutions to be checked through website
- Streptavidine poly HRP (Thermo Scientific, Prod # N200)
- TMB Substrate (Thermo Scientific Prod #3402)
- 1N H2SO4 (Alfa Aesar, LOT F040011 / Stock 35655).

Preparation of the wash buffer:

MilliQ water + 0.9% NaCl + 0.05% Tween20 per plate (9g NaCl & 500uL Tween 20 in 1L of water). The prepared wash buffer can be kept in the fridge for up to 4 weeks.

Preparation of blocking buffer & working buffer:

PBS pH=7.8 + 0.5% BSA (0.5mL BSA in 100mL PBS). Can be kept for 24 hours. If you wish to keep it for up to 2 weeks, increase the amount of BSA to 5%. (30ml PBS 7.8 + non-sterile 500uL BSA).

To coat the plate:

After the capture antibody incubation overnight, take it out and pipette 300uL on block buffer. Do not wash it. Take the excess out by flipping the plate. Dry it upside down in a filter paper. Keep it at 4°C for up to 4 weeks.

6 OPERATING INSTRUCTION

Day 1

1. Capture antibody dilution (1:1000, 10uL in 10ml) in PBS (pH=7.8).
   Correct dilution has to be checked for each cytokine.
2. Coat 96 well flat bottom plates with 100uL of the diluted capture antibody.
   Seal the plate and incubate over night at 4 C.

Day 2

3. Prepare wash and blocking buffer.
4. Empty wells by tapping it on a paper filter quickly. Add block buffer 300uL and incubate for 2h in room temperature. In the meantime prepare the standards.
5. Wash the plates 4 times with 300uL of washbuffer per well, using the plate whasher machine.
6. Prepare the standard curve in the same CF1 you have used for your culture by diluting the stock (500, 250, 125, 62.5, 31.25, 15.75, 7.8, 3.9, 1.95, 0) to make 9 points plus blank in duplicate. Dilute it in your samples in 10ml of working buffer.
7. Prepare the detection antibody in working buffer (1:500), the recombinant human cytokines and your samples to the plates.
8. Pipette 100uL of samples and the stardard curve in duplicates to the appropriate wells.

9. Add 50uL of the diluted detection antibody to each well.
10. Incubate 2hr on a plate shaker at room temperature.
11. Wash the plate 4 times with 300uL of wash buffer per well, using the plate washer.
12. Dilute the streptavidin poly HRP 1:15000 (66uL in 10ml) in working buffer and add 100uL to each well.
13. Incubate 30 minutes on a plate shaker at room temperature.
14. Put the substrate at room temperature.
15. Wash the plates 4 times with 300uL of wash buffer per well, using the plate washer machine. Make sure to tip it well!
16. Just before use, prepare TMB solution by mixing substrate A and peroxidise solution substrate B (1:1). Then pipette 100uL per well. For an entire plate mix 5mL substrate A + 5mL substrate B.
17. Incubate on a plate shaker at room temperature until you see a clear standard curve (or when the BLANK will start to be slightly blue).
18. Stop the reaction with 100uL of 1M H_2SO_4 per well.
19. Read it at 450nm with a background of 620nm with an ELISA reader.

7 REMARKS

- BSA30%, Capture Ab, Detection Ab, TMB are kept in the fridge.
- Recombinant IL-6 is diluted 1:10000 ng/ml (5 ml R&D in 50 ml WB) for standard curve. The standard curve aliquots (700uL) are kept at -80°C labelled SC24/05/11.
- Streptavidin is diluted 1:100 (250uL in 25ml PBS), aliquoted (75uL) and kept at -20°C.

8 REFERENCES

9 APPENDICES

Appendix-M RNA Isolation from PAXgene

RNA ISOLATION FROM PAXGENE

1. Defrost samples for 3 hours before beginning processing.

2. Invert PAXgene tubes twice and then centrifuge for 15 min at 3000x g with break.

3. Remove the supernatant by decanting into waste bottle containing trigene.

4. Add 4 ml RNase-free water to the pellet, and close the tube using a fresh secondary Hemogard closure.

5. Vortex until the pellet is visibly dissolved, and centrifuge for 15 min at 3000 x g with break. Remove the entire supernatant by decanting and discard.

6. Add 350 µl Buffer BM1, and pipette up and down until the pellet is visibly dissolved then pipette the sample into a 1.5 ml eppendorf tube.

7. Add 300 µl Buffer BM2 and 40 µl proteinase K. Mix by vortexing for 5 s, and incubate for 10 min at 55°C in an incubator (PSDL space).

8. Pipette (use 700 µl setting) the sample into a PAXgene Shredder spin column (lilac) placed in a 2 ml processing tube, and centrifuge for 3 min at full speed 16,000 x g (PSDL space).

9. Remove inner lilac part. Carefully transfer the entire supernatant of the flow-through from the PAXgene Shredder spin column to a new 1.5 ml eppendorf tube without disturbing the pellet in the processing tube. (Re centrifuge is pellet is disturbed).

10. Add 700 µl of isopropanol (100%, purity grade p.a.), and mix by vortexing for 15seconds.

11. Pipette 700 µl sample into the PAXgene RNA spin column (pink) placed in a 2 ml processing tube. Close the lid gently, and centrifuge for 1 min at 16,000 x g.

12. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.

13. Pipette the remaining sample into the PAXgene RNA spin column (pink). Close the lid gently, and centrifuge for 1 min at 16,000 x g. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.

14. Add 350 µl Buffer BM3 to the PAXgene RNA spin column. Close the lid gently, and centrifuge for 15 s at 16,000 x g. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.

15. Add 10 µl DNase I stock solution to 70 µl Buffer RDD in a 1.5 ml microcentrifuge tube (this is per sample so multiply amounts by number of samples). Mix by pipetting up and down or by gently flicking the tube.
Note: DNase I is especially sensitive to physical denaturation. Do not vortex.

16. Pipette the DNase I incubation mix (80 µl) **directly onto the PAXgene RNA spin column membrane** (without touching the membrane), and incubate on the bench top (20–30°C) for 15 min.

17. Add 350 µl Buffer BM3 to the PAXgene RNA spin column. Close the lid gently, and centrifuge for 15 s at 16,000 x g. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.

18. Add 500 µl Buffer BM4 to the PAXgene RNA spin column. Close the lid gently, and centrifuge for 15 s at 16,000 x g. Discard the flow-through. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.

19. Add another 500 µl Buffer BM4 to the PAXgene RNA spin column. Close the lid gently, and centrifuge for 2 min at 16,000 x g.

20. Discard the processing tube containing flow-through, and place the PAXgene RNA spin column in a new 2 ml processing tube (supplied). Centrifuge at 16,000 x g for 1 min. **It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions.**

21. Discard the processing tube containing flow-through. Place the PAXgene RNA spin column in a new 1.5 ml microcentrifuge tube, and pipette 40 µl Buffer BR5 **directly onto the PAXgene RNA spin column membrane** (without touching the membrane). Close the lid gently, and centrifuge for 1 min at 16,000 x g to elute the RNA.

22. Collect ice and for the following steps keep samples on ice.

23. Collect the flow-through and pass through the spin column again by pipetting **directly onto the PAXgene RNA spin column membrane** (without touching the membrane).